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# OZENA AND DISTEMPER: COMPARATIVE STUDY OF COCCOBACILLUS FOETIDUS-OZAENAE AND BACILLUS BRONCHISEPTICUS.

N. S. FERRY AND ARLYLE NOBLE

*From the Research Laboratory of Parke, Davis and Company, Detroit, Michigan*

Received for publication November 15, 1917

The question of the relationship of ozena to distemper has been frequently discussed, both in print and otherwise. Many observers consider the dog a carrier of an organism common to both diseases; and the patients themselves are often of the same opinion as they will frequently volunteer information bearing on their association with dogs, special emphasis being laid on a coincident infection of the animal with distemper sometime in the early part of the disease.

Perez (1913) carried out some experimental work along this line and suggested that the infection in ozena probably originates from dogs, and, as a result of his work, cultures from these animals were included in an ozena vaccine upon which Hofer and Kofler (1914), collaborators of Perez, were experimenting.

Horn and Victors (1916) in this country, considering *Coccobacillus foetidus-ozaenae* (Perez) as the principal etiological factor in ozena and *Bacillus bronchisepticus* (Ferry) as the cause of distemper, have even attempted to prove a relationship between the above mentioned organisms and claim to have found a positive complement fixation between them. The question, however was left in rather an unsettled condition as may be seen by the following statement made by them:

"*Bacillus bronchisepticus*, exhaustively studied by Ferry, M'Gowan, Torrey and Rahe, and determined by them as being the specific organism of canine distemper, morphologically almost identical and biologically in many instances similar to the Perez organism, and the

advanced hypothesis that the infection of fetid ozena is carried by dogs, presents an interesting complex."

It is not the province of this paper to discuss nor to attempt to offer any proof pro or con as to the etiology of distemper in the dog or of ozena in the human subject (that phase of the question having been taken up by the several authors elsewhere); but rather to attempt to answer the question, as to the relationship of Perez' organism to *B. bronchisepticus*, which has been so significantly brought forward by Horn and Victors.

If Perez' bacillus is the cause of ozena and it is demonstrated to be the same as, or closely related to, *B. bronchisepticus*, an extremely important point has been established and its bearing on the prophylaxis and management of ozena can hardly be estimated at the present time, since distemper is most widely distributed among dogs and other small animals, especially laboratory animals, such as the rabbit and guinea pig. Accordingly, the authors, using several typical strains of both organisms, have carried on a large number of comparative morphological, cultural and serological tests in an attempt to determine, if possible, the characteristics common or perhaps, to be more exact, not common to both organisms.

For the morphological and cultural characteristics of *B. bronchisepticus* those originally published by one of us (Ferry, 1910, 1911) and corroborated by M'Gowan (1911) and Torrey and Rahe (1913) are taken as characteristic; and for *Coccobacillus foetidus-ozaenae* only those described by Ward (1917) are considered, as they apply directly to the strains under discussion in this work. Unfortunately, there does not seem to be a unanimity of opinion among writers relative to all reactions of this organism. The question of motility seems to be still undecided. The four strains under discussion, however, were found by the authors to be motile, although not so progressively active as *B. bronchisepticus*. In this instance the authors do not agree with Ward, who claims that the European strains are non-motile.

## SUMMARY OF CULTURAL REACTIONS

1. *B. ozaenae* may be readily differentiated from *B. bronchisepticus* by any one of the following reactions; by growth on potato, on Loeffler's blood serum and in litmus milk, by the fermentation reaction in glucose media and by the indol reaction in Dunham's solution.

2. Less important differentiating characteristics are found in morphology, motility, colony formation and odor.

*Differentiating cultural characteristics*

| | PEREZ' BACILLUS | B. BRONCHISEPTICUS |
|---|---|---|
| Morphology | Small coccoid bacillus, no filaments | Small narrow bipolar bacillus. Filaments in liquid media |
| Motility | Sluggish | Active |
| Colony | Old colony, lobate | Round, entire |
| Loeffler's blood serum | No proteolysis, cream to greenish yellow | No proteolysis, old cultures tan color |
| Potato | Limited, faintly yellow | Spreading, tan |
| Plain broth | Uniform turbidity, characteristic nauseating odor | Uniform turbidity, odor of stale bread |
| Litmus milk | Small amount of acid | Decided alkalinity |
| Dunham's | Indol positive | Indol negative |
| Fermentation | | |
| Glucose | Acid—gas | Alkaline—no gas |

## AGGLUTINATION REACTIONS WITH B. BRONCHISEPTICUS AND PEREZ' BACILLUS

Rabbits have been treated with vaccines of the following cultures of *B. bronchisepticus* and Perez' Bacillus and the sera obtained for the purpose of making cross agglutination tests between these two organisms.

*B. bronchisepticus* no. 36, from dog
*B. bronchisepticus* no. 123, from monkey } ..... Isolated by N. S. F.
*B. bronchisepticus* from human

Perez' Bacillus no. 1 ..... Isolated by Ward

Perez' Bacillus no. 2, "Hofer" strain
Perez' Bacillus no. 3, "Vienna" strain } ..... European strains

Perez' Bacillus no. 4 ..... Isolated by Ward

*Vaccines.* Each organism was transplanted daily for several days on plain agar; then twenty-four hour growths were washed off in 0.85 per cent salt solution plus 0.2 per cent trikresol (5 cc. per culture). Each vaccine was thoroughly shaken in a mechanical shaker and two days later tested for sterility.

*Production of antisera.* Before the rabbits were injected each was bled from an artery in the ear and the serum tested for ag-

TABLE 1

| DILUTIONS | SERUM FROM RABBIT 32, TREATED WITH B. BRONCHISEPTICUS NO. 36 (DOG). AGAINST SUSPENSIONS OF: | | | | | | |
|---|---|---|---|---|---|---|---|
| | *B. bronchisepticus* | | | Perez' Bacillus | | | |
| | No. 36 (dog) | No. 123 (monkey) | Human | No. 1 | No. 2 | No. 3 | No. 4 |
| 1-10 | +++ | +++ | +++ | – | – | – | – |
| 1-20 | +++ | +++ | +++ | – | – | – | – |
| 1-40 | +++ | +++ | +++ | – | – | – | – |
| 1-80 | +++ | +++ | +++ | – | – | – | – |
| 1-200 | +++ | +++ | +++ | – | – | – | – |
| 1-400 | +++ | +++ | +++ | – | – | – | – |
| 1-800 | +++ | +++ | +++ | – | – | – | – |
| 1-1600 | +++ | +++ | +++ | – | – | – | – |
| 1-2000 | +++ | +++ | +++ | – | – | – | – |
| 1-3200 | +++ | +++ | +++ | – | – | – | – |
| 1-6400 | + | + | + | – | – | – | – |
| 1-10000 | – | – | – | – | – | – | – |
| 1-20000 | – | – | – | – | – | – | – |
| Control | – | – | – | – | – | – | – |

+++ = Complete agglutination with all organisms clumped and fluid clear.
++ = Partial agglutination, with marked clumping but fluid not cleared up.
+ = Slight agglutination but still with positive clumping.
– = No clumping and no clearing.

glutinins against both *B. bronchisepticus* and Perez' Bacillus. No rabbit showed an agglutination titre of above 1 in 20 against either organism.

Each rabbit received three intravenous injections of 0.5, 1.0 and 2.0 cc. of killed vaccine three days apart, and was bled to death on the fourth day after the last dose. To the sera was added 0.2 per cent trikresol.

*Preparation of suspensions for agglutination tests.* Each organism was transplanted daily, on plain agar, for ten days; twenty-four hour growths planted on plain agar in whiskey flasks; incubated eighteen hours; growths washed off in physiologic salt solution plus 0.5 per cent formalin. The suspensions were shaken for two hours; two days later tested for sterility; then filtered three times through filter paper. Each was later diluted with salt solution plus 0.5 per cent formalin to correspond in density to our standard suspension of *B. bronchisepticus*,

TABLE 2

| DILUTIONS | SERUM FROM RABBIT 36, TREATED WITH PEREZ' BACILLUS NO. 1. AGAINST SUSPENSIONS OF: | | | | | | |
|---|---|---|---|---|---|---|---|
| | Perez' Bacillus | | | | *B. bronchisepticus* | | |
| | No. 1 | No. 2 | No. 3 | No. 4 | No. 36 (dog) | No. 123 (monkey) | Human |
| 1–10 | ++ | +++ | ++ | ++ | + | + | + |
| 1–20 | +++ | +++ | +++ | +++ | – | – | – |
| 1–40 | +++ | +++ | +++ | +++ | – | – | – |
| 1–80 | +++ | +++ | +++ | +++ | – | – | – |
| 1–200 | +++ | +++ | +++ | +++ | – | – | – |
| 1–400 | +++ | +++ | +++ | +++ | – | – | – |
| 1–800 | +++ | +++ | +++ | +++ | – | – | – |
| 1–1600 | +++ | +++ | +++ | +++ | – | – | – |
| 1–2000 | +++ | +++ | +++ | +++ | – | – | – |
| 1–3200 | +++ | ++ | +++ | +++ | – | – | – |
| 1–6400 | + | + | – | ++ | – | – | – |
| 1–10000 | – | – | – | – | – | – | – |
| 1–20000 | – | – | – | – | – | – | – |
| Control | – | – | – | – | – | – | – |

which contains about 2,000,000,000 organisms per cubic centimeter. Perfectly homogenous suspensions of all the strains used were produced by this method.

*Agglutination tests.* The serum was diluted with physiologic salt solution and each tube contained 0.5 cc. suspension plus 0.5 cc. diluted serum. The tests were incubated at 37°C. and readings made at the end of twenty-four hours.

Tables 1, 2 and 3 show the results of the agglutination experiments.

TABLE 3

| ANTISERA | | CROSS AGGLUTINATION TESTS WITH B. BRONCHISEPTICUS AND PEREZ' BACILLUS. SUSPENSIONS | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *B. bronchisepticus* | | | Perez' Bacillus | | | |
| | | No. 36 (dog) | No. 123 (monkey) | Human | No. 1 | No. 2 | No. 3 | No. 4 |
| *B. bronchisepticus* | | | | | | | | |
| No. 36 | Rabbit 32 | **1–6400** | | | – | – | – | – |
| | Rabbit 2 | **1–10000** | 1–20000 | 1–10000 | – | – | – | – |
| No. 123 | Rabbit 33 | | **1–6400** | | – | – | – | – |
| | Rabbit 34 | | **1–6400** | | – | – | – | – |
| | Rabbit 4 | 1–10000 | **1–10000** | 1–20000 | | | | |
| Human | Rabbit 5 | | | **1–6400** | – | – | – | – |
| | Rabbit 6 | 1–10000 | 1–10000 | **1–10000** | – | – | – | – |
| *Perez' Bacillus* | | | | | | | | |
| No. 1 | Rabbit 36 | 1–10 | 1–10 | 1–10 | **1–6400** | 1–6400 | 1–3200 | 1–6400 |
| | Rabbit 37 | – | – | – | **1–6400** | | | |
| No. 2 | Rabbit 38 | – | 1–10 | – | 1–10000 | **1–6400** | 1–6400 | 1–10000 |
| | Rabbit 39 | – | – | – | | **1–3200** | | |
| No. 3 | Rabbit 40 | – | – | – | 1–10000 | 1–6400 | **1–10000** | 1–10000 |
| | Rabbit 41 | 1–10 | – | – | | | **1–6400** | |
| No. 4 | Rabbit 42 | 1–10 | 1–20 | 1–10 | 1–6400 | 1–6400 | 1–6400 | **1–10000** |
| | Rabbit 43 | 1–20 | 1–20 | 1–20 | | | | **1–10000** |

## SUMMARY OF AGGLUTINATION EXPERIMENTS

1. Sera of high agglutination titre (1–3200 to 1–10000) were produced in rabbits by three intravenous injections of killed unheated vaccines of *B. bronchisepticus* and of Perez' Bacillus

2. All the strains of Perez' Bacillus used were identical by agglutination test—each antiserum agglutinating all strains equally well.

3. All strains of *B. bronchisepticus* were identical.

4. There was no cross agglutination between *B. bronchisepticus* and Perez' Bacillus.

## COMPLEMENT FIXATION REACTIONS WITH B. BRONCHISEPTICUS AND PEREZ' BACILLUS

Complement fixation tests were made with the same antisera as used for the agglutination reactions.

*Technic.* The volume of all the complement fixation tests and all titrations was 4.5 cc. The method in general was that given by Kolmer in "Infection, Immunity and Specific Therapy."

For the hemolytic system, *sheep corpuscles in 5 per cent solution* (the blood was defibrinated with beads, measured, washed five times in salt solution, diluted back to original volume and 5 cc. of this used in 100 cc. salt solution), *rabbit amboceptor*, and *guinea-pig complement* in a 1 in 20 dilution (serum from at least two pigs being mixed) were used. The amboceptor was titrated daily with each complement and corpuscle suspension and that amount which showed just complete hemolysis in one hour at 37°C. was taken as the unit. In the tests one and a half times the unit was used.

Each antigen was titrated for its anticomplementary and its antigenic unit. In the tests, one-half to one-fourth the anticomplementary unit was used.

Each antiserum was tested to determine the smallest amount which, with its homologous antigen, completely inhibited hemolysis.

*The test.* Antigen plus serum plus complement was incubated one hour at 37°C. then amboceptor and cells were added and incubated from one to one and one-half hour depending on the antigen controls. The tests were then placed in the ice-chest and read the following morning.

*Antigens.* *B. bronchisepticus* and Perez' Bacillus were transplanted daily for several days, then planted on plain agar in whiskey flasks; incubated for twenty-four hours at 37°C.; the growths washed off in sterile distilled water (about 50 cc. per flask); shaken in a mechanical shaker for forty-eight hours; brought up in a water bath to 56°C. and incubated at that temperature over night; the antigens were then filtered through asbestos and to nine parts of clear filtrate one part of 8.5 per

cent salt solution plus 5 per cent formalin was added. The antigens were then kept in the ice chest.

*Cross titrations.* Cross titrations were made between the several strains of *B. bronchisepticus*, the several strains of Perez' Bacillus and between *B. bronchisepticus* and Perez' Bacillus.

Each serum was tested against each of the seven antigens and on the same day, all the tests being run parallel.

Tables 4, 5 and 6 show the results of the complement fixation tests.

TABLE 4

| AMOUNT OF SERUM | SERUM FROM RABBIT 32, TREATED WITH B. BRONCHISEPTICUS NO. 36 (DOG). AGAINST ANTIGENS OF: | | | | | | |
|---|---|---|---|---|---|---|---|
| | *B. bronchisepticus* | | | Perez' Bacillus | | | |
| | No. 36 (dog) | No. 123 (monkey) | Human | No 1 | No. 2 | No. 3 | No. 4 |
| cc. | | | | | | | |
| 0.1 | C. I. | C. I. | C. I. | C. H. | C. H. | C. H. | C. H. |
| 0.05 | C. I. | C. I. | C. I. | C. H. | C. H. | C. H. | C. H. |
| 0.01 | C. I. | C. I. | C. I. | C. H. | C. H. | C. H. | C. H. |
| 0.008 | C. I. | C. I. | C. I. | C. H. | C. H. | C. H. | C. H. |
| 0.005 | C. I. | C. I. | C. I. | C. H. | C. H. | C. H. | C. H. |
| 0.004 | C. I. | Marked I | Marked I | C. H. | C. H. | C. H. | C. H. |
| 0.003 | C. I. | Marked I | Marked I | C. H. | C. H. | C. H. | C. H. |
| 0.002 | C. I. | Slight I | Slight I | C. H. | C. H. | C. H. | C. H. |
| 0.001 | Slight I. | C. H. | C. H. | C. H. | C. H. | C. H. | C. H. |
| Controls: | | | | | | | |
| Antigen..... | C. H. | C. H. | C. H. | C. H. | C. H. | C. H. | C. H. |
| Serum..... | C. H. | | | | | | |
| Hemolytic..... | C. H. | | | | | | |
| Corpuscle..... | No. H. | | | | | | |

C. H. = Complete hemolysis.
C. I. = Complete inhibition of hemolysis.

## SUMMARY OF COMPLEMENT FIXATION TESTS

1. Sera of high complement-fixing titre were produced in rabbits by three intravenous injections of killed unheated suspensions of *B. bronchisepticus* and Perez' Bacillus.

2. The four strains of Perez' Bacillus gave identical complement fixation reactions.

TABLE 5

| AMOUNT OF SERUM | SERUM FROM RABBIT 36, TREATED WITH PEREZ' BACILLUS NO. 1, AGAINST ANTIGENS OF: | | | | | | |
|---|---|---|---|---|---|---|---|
| | Perez' Bacillus | | | | *B. bronchisepticus* | | |
| | No. 1 | No. 2 | No. 3 | No. 4 | No. 36 (dog) | No. 123 (monkey) | Human |
| cc. | | | | | | | |
| 0.01 | C. I. | C. I. | C. I. | C. I. | C. H. | C. H. | C. H |
| 0.05 | C. I. | C. I. | C. I. | C. I. | C. H. | C. H. | C. H. |
| 0.01 | C. I. | C. I. | C. I. | C. I. | C. H. | C. H. | C. H. |
| 0.008 | C. I. | C. I. | C. I. | C. I. | C. H. | C. H. | C. H. |
| 0.005 | C. I. | C. I. | C. I. | C. I. | C. H. | C. H. | C. H. |
| 0.004 | C. I. | C. I. | C. I. | C. I. | C. H. | C. H. | C. H. |
| 0.003 | C. I. | C. I. | C. I. | C. I. | C. H. | C. H. | C. H. |
| 0.002 | Marked I. | Marked I. | Marked I. | Marked I. | C. H. | C. H. | C. H. |
| 0.001 | Slight I. | C. H. | Slight I. | C. H. | C. H. | C. H. | C. H |
| Controls: | | | | | | | |
| Antigen..... | C. H. | C. H. | C. H. | C. H. | C. H. | C. H. | C. H |
| Serum..... | C. H. | | | | | | |
| Hemolytic..... | C. H. | | | | | | |
| Corpuscle..... | No H. | | | | | | |

TABLE 6

*Cross complement fixation tests with B. bronchisepticus and Perez' Bacillus*

| ANTISERA | ANTIGENS | | | | | | |
|---|---|---|---|---|---|---|---|
| | *B. bronchisepticus* | | | Perez' Bacillus | | | |
| | No. 36 (dog) | No. 123 (monkey) | Human | No. 1 | No. 2 | No. 3 | No. 4 |
| | cc. | cc. | cc. | cc. | cc. | cc. | cc. |
| *B. bronchisepticus* | | | | | | | |
| No. 36 Rabbit 32..... | **0.002** | 0.005 | 0.005 | — | — | — | — |
| No. 36 Rabbit 2..... | **0.004** | 0.008 | 0.008 | — | — | — | — |
| No. 123 Rabbit 33..... | 0.008 | **0.003** | 0.003 | — | — | — | — |
| No. 123 Rabbit 34..... | 0.05 | **0.008** | 0.008 | — | — | — | — |
| *Human* | | | | | | | |
| Human Rabbit 5..... | 0.05 | 0.003 | **0.003** | — | — | — | — |
| Human Rabbit 6..... | 0.05 | 0.005 | **0.005** | — | — | — | — |
| *Perez' Bacillus* | | | | | | | |
| No. 1, Rabbit 36..... | — | — | — | **0.003** | 0.003 | 0.003 | 0.003 |
| No. 2, Rabbit 38..... | — | — | — | 0.003 | **0.003** | 0.003 | 0.003 |
| No. 3, Rabbit 40..... | — | — | — | 0.003 | 0.003 | **0.003** | 0.003 |
| No. 4. Rabbit 42..... | — | — | — | 0.002 | 0.002 | 0.002 | **0.002** |

3. The human and monkey strains of *B. bronchisepticus* gave identical complement fixation reactions, but these two strains cross-fixed with the dog strain only with larger amounts of serum.

4. Immune sera of *B. bronchisepticus* did not cross-fix with antigens of Perez' Bacillus.

5. Immune sera of Perez' Bacillus did not cross-fix with antigens of *B. bronchisepticus*.

## CONCLUSIONS

According to the cultural reactions and the agglutination and complement fixation tests there appear to be no reasons why Perez' Bacillus should be confused with *B. bronchisepticus*. The organisms can be differentiated from each other by any one or more of the above methods.

The results of Horn and Victors with the complement fixation test have not been corroborated by us.

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# STUDIES ON THE PROTEOLYTIC ENZYMES OF SOIL FUNGI AND ACTINOMYCETES[1]

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The proteolytic enzymes of microörganisms have been studied by a number of investigators; but most of the data obtained are merely qualitative in nature. A full bibliography on the investigations concerning bacterial enzymes can be found in the work of Fuhrman (1907); the investigations of the enzymes of molds have been reviewed by Wehmer (1907) and by Dox (1910). Mention will here be made only of those investigations which have a direct bearing upon the work at hand.

## ORGANISMS USED IN THE PRESENT STUDY

A number of organisms isolated from different soils were used for this work, although the greatest amount of work was devoted to the first named organism.

*Aspergillus niger* van Tieghem. This organism was isolated from a sandy loam soil on the College Farm, at New Brunswick, New Jersey. Methods of isolation and the description of soils, for this as well as for the following organisms, can be found in another place (Waksman, 1916). This organism was selected for the work because many of the previous investigations on enzymes of molds have been conducted with it. Since its identification is not difficult, we may suppose that previous investigators used the organism originally described by van Tieghem, although, as was recently shown by Thom (1916), the organism

[1] The data presented in this paper form Part II of the dissertation presented by the author for the degree of Doctor of Philosophy, University of California, December, 1917.

belongs to the group of black Aspergilli which have not been well differentiated before.

*Aspergillus ochraceus* Wilhelm. A preliminary description of this organism has appeared in another place (Waksman, 1916), as C. 19. The organism revealed peculiar metabolic processes and a number of investigations were conducted with it.

*Aspergillus fuscus* Schiemann.

*Aspergillus clavatus* Desmazières.

*Citromyces glaber* Wehmer.

*Penicillium chrysogenum* Thom.

*Actinomyces griseus* (Krainsky) Waksman and Curtis.

*Actinomyces violaceus-ruber* Waksman and Curtis.

*Actinomyces* sp. 101 (*virido-chromogenus?*) Krainsky Waksman and Curtis. A full description of this organism will soon appear.

*Actinomyces californicus* Waksman and Curtis.

## METHODS USED IN THE INVESTIGATION

The organisms were grown on Czapek's solution composed as follows:

| | | |
|---|---|---|
| $NaNO_3$ | 2.0 | grams |
| $K_2HPO_4$ | 1.0 | gram |
| KCl | 0.5 | gram |
| $MgSO_4$ | 0.5 | gram |
| $FeSO_4$ | 0.01 | gram |
| Cane sugar | 30.0 | grams |
| Distilled water | 1000 | cc. |

In addition another medium was made up by substituting 20 grams peptone for the 2 grams $NaNO_3$ per liter. This medium is called Peptone-Czapek solution. The media were distributed in 100 cc. portions in 200 cc. Erlenmeyer flasks, plugged and sterilized for fifteen minutes at 15 pounds pressure. The flasks were then inoculated and incubated at 28°C. At the end of the proper incubation period, the cultures were filtered through No. 584 folded Schleicher & Schüll filters. The filtrate was used for the study of the exoenzymes; while the mycelium was washed with distilled water, and then treated by the acetone method, as used by Doy (1910), Scales (1914), and others. Twenty

cubic centimeters of the liquid or a portion of the treated mycelium, equivalent to 0.5 to 0.8 gram of the dried substance per 100 cc. of 1 per cent solution of substratum were used. In several cases Grübler's trypsin and pepsin were used for comparison of the enzyme activities. The protein solutions were heated to boiling so as to kill the proteolytic enzymes that they might possibly contain. In all cases a check was made by boiling the protein with the added enzyme, then incubating it in parallel with the enzyme cultures. The proper reaction was always obtained by the use of 0.1 N KOH or HCl. Both chloroform and toluene were used to keep the solutions sterile. The substrata, plus enzyme, as well as the blanks, were incubated at 37°C. After the proper incubation period, 2 cc. portions of the solution were used for the determination of amino nitrogen by the use of the micro-apparatus of Van Slyke (1911, 1913). Duplicate determinations were made, but these always checked very well, so that only the averages are given in the tables. The amino nitrogen content was always figured back to 100 cc. of solution.

As substrata the following proteins were used: (1) Merck's peptone; (2) casein prepared after Hammarsten, each gram at first being dissolved in 8 cc. of 0.1N KOH, then brought to the proper reaction by the use of 0.1N HCl; (3) crystalline egg-albumen prepared from fresh eggs using the method of Hopkins and Pincus (1898); (4) Merck's fibrin, 1 gram per 100 cc. of water. The concentration of all the substrata was made up in such a manner as to constitute 1 per cent solutions upon the addition of the proper amount of enzyme.

It appeared desirable to obtain, first, an insight into the nature of the exo- and endoenzymes of several microörganisms. Dox (1910) claimed that the same enzyme may, in the earlier stages of growth of the organism, function within the cell, and later be liberated into the substratum as an extracellular enzyme. Abderhalden and Pringsheim (1910) maintained that the absence of enzymes in the liquid obtained by the Buchner method cannot serve as a criterion for the absence of particular enzymes; the mycelium itself from the culture has to be studied for the presence of the enzyme, which may not have diffused into the liquid medium.

A number of organisms were grown on Czapek's solution, for a period of eighteen to sixty days, the slower growing organisms, such as the actinomycetes, requiring a longer incubation period. At the end of that period, the cultures were filtered and the mycelium treated by the acetone method. This method consists in washing the mycelium a few times with distilled water, then drying it between filter paper, cutting it into fine pieces, and covering it for ten minutes with acetone; the mycelium is then dried again and covered for three minutes with ether, and finally dried over sulfuric acid at 35 to 40°C. One per cent pep-

TABLE 1

*Action of exo- and endoenzymes of microörganisms upon casein and peptone*

| ORGANISMS USED | AGE OF CULTURE | MILLIGRAMS OF $NH_2$-N PER 100 CC. OF SOLUTION | | | |
|---|---|---|---|---|---|
| | | 1 per cent peptone | | 1 per cent casein | |
| | | Exoenzyme | Endoenzyme | Exoenzyme | Endoenzyme |
| | *days* | | | | |
| Control | | 21.36 | 20.26 | 7.26 | 6.20 |
| *A. niger* | 18 | 22.11 | 41.32 | 10.48 | 29.10 |
| *A. ochraceus* | 18 | 26.19 | 47.72 | 19.20 | 37.83 |
| *Citr. glaber* | 18 | 24.44 | 47.14 | 17.46 | 55.29 |
| *P. chrysogenum* | 8 | 50.40 | | 16.90 | |
| *P. chrysogenum* | 60 | 59.36 | 62.27 | 70.42 | 68.09 |
| *Act. griseus* | 60 | 37.25 | 27.35 | 52.38 | 34.92 |
| *Act. sp.* 101 | 60 | 50.63 | 37.25 | 52.38 | 75.66 |
| *Act. violaceus-ruber* | 60 | 20.95 | 38.76 | 55.29 | 43.65 |
| Trypsin: 200 mgm. | | 47.12 | | 62.12 | |

tone and casein solutions, neutral to litmus, were used as substrata. Fifty milligrams of treated actinomyces mycelium and 800 mgm. of fungus mycelium were taken for the inoculation of each flask for the study of the endoenzymes. Determinations of the amino nitrogen formed as a result of the action of the enzyme upon the substratum were made at the end of four days. The blank was subtracted from each determination.

It is seen from table I that both the exo- and endoenzymes of the microörganisms studied, when these organisms have been grown on a non-protein medium, can split casein and peptone

to amino-acids or to compounds containing amino nitrogen. The action of the endoenzymes was stronger than that of the exoenzymes, in the case of the fungi, but, in the case of the actinomycetes, the reverse held true in most instances; this is probably due to the fact that the treated mycelium containing the endoenzymes of the actinomycetes was added in much smaller quantities than that of the fungi since the growth of the former is very scant. It is interesting to note one thing in the above table, namely that *P. chrysogenum* and *Act.* sp. 101, which were found to produce more amino nitrogen than ammonia when grown in a peptone solution (Waksman, 1918), produced enzymes, which have a strong action upon the proteins, while the other organisms, which produced more ammonia than amino nitrogen in the peptone solution, gave rise to much weaker enzymes; this fact may throw some light upon the problem of protein decomposition by microörganisms.

It was pointed out by Kendall and his associates (1915) that the sugar content of the medium has a decided influence upon the production of proteolytic enzymes by bacteria. To see whether the same thing holds true with fungi, the enzymes obtained from *A. niger* grown for fifteen days on the Peptone-Czapek solution containing 0, 1, 3, 5 and 20 per cent of sugar were used.

It is seen from table 2 that the sugar content of the medium has no decided influence upon the production of proteolytic enzymes by *A. niger*. Where sugar was present in the original medium just as much protein is split by both enzymes and, in some cases, even more than by the enzymes of the organism grown on the sugar free medium. The liquefaction of gelatin by the enzymes obtained from the above cultures did not appear to be affected by the presence of sugar in the original medium. The bacteria seem to behave differently from the fungi in this respect.

It was found advisable to determine first the proper reaction which would be optimum for the activities of the enzymes and also the proper age of the cultures, when the activities of the exoenzymes would be at a maximum. Several organisms were

grown on Czapek's and Peptone-Czapek solution and the filtrates only used for experiment.

The results brought out in the above experiment show a distinct difference between the activities of microbial and animal proteolytic enzymes. In most instances the substratum neutral to litmus contained a larger quantity of amino nitrogen; at least the more alkaline reaction did not prove more favorable for the enzyme activities of microörganisms than the more acid reaction. This would seem to point to several conclusions: either the organisms produce a mixture of two enzymes which are peptic and ereptic in nature, or, if the enzyme is of a tryptic

TABLE 2

*Influence of sugar content of the medium upon the proteolytic activities of the exo- and endoenzymes of A. niger.*

| SUGAR CONTENT OF MEDIUM | MILLIGRAMS OF $NH_2$=N PER 100 CC. OF SOLUTION | | | |
|---|---|---|---|---|
| | 1 per cent peptone | | 1 per cent casein | |
| | Exoenzyme | Endoenzyme | Exoenzyme | Endoenzyme |
| Control | 21.32 | 20.30 | 7.28 | 6.20 |
| 0 | 45.24 | 41.84 | 45.00 | 39.44 |
| 1 per cent | 46.40 | 44.72 | 54.56 | 54.00 |
| 3 per cent | 47.60 | 50.56 | 43.44 | 54.56 |
| 5 per cent | 38.86 | 45.80 | 25.38 | 55.06 |
| 20 per cent | 44.24 | 52.78 | 38.28 | 56.32 |
| Trypsin: 200 mgm. | 46.68 | | 56.56 | |

nature, it is much less sensitive to an acid reaction than animal trypsins are. Finally, the enzymes produced by the microorganisms studied may be of a nature entirely different from that of the animal proteolytic enzymes, and may be comparable to those found by Vines (1900–1910) in plants; the range of their optimum reaction is certainly greater than that of corresponding animal enzymes. *A. niger* and *A. fuscus*, both closely related species, produced, in the case of casein, less amino nitrogen with the more acid reaction, and the same thing held true for the enzymes of *A. niger* and *Act.* sp. 101 grown on the peptone-containing medium.

When the activities of the enzymes are compared with regard to the age of the culture, we find that the five-day-old cultures gave, on the average, a higher amino nitrogen production from both peptone and casein, and with both reactions. This is not fully in accord with the idea of Dox (1910) that in the older cultures the enzymes are excreted into the substratum, and the liquid therefore becomes richer in enzymes. It is likely however that the weakness of the enzymes in the older cultures may be due to an accumulation of acids or other waste products in the media, which might influence injuriously the activities of the enzymes.

That the enzymes produced by the organisms studied are not merely pepsins can be seen by glancing at table 3. The peptone was split just as well as the casein, giving amino-acids and other simple amino-nitrogen-containing compounds, the identity of which has not been established with certainty.

The culture medium, on which the organisms were grown, seems to have a decided influence upon the amount of enzymes present in the medium. The peptone-containing medium gave in all instances a much stronger proteolytic action than the peptone-free medium; but even on the peptone-free media proteolytic enzymes were also produced. This points to the fact that the variation in enzyme production by microörganisms on different media is not of a qualitative but of a quantitative character, as has already been demonstrated by other investigators.

Small quantities of ammonia amounting to a few milligrams of nitrogen per 50 cc. of solution were found in the substrata inoculated with the enzymes; this is probably due to the action of the desamidasing enzymes, as was shown by Shibata (1902), Pringsheim (1908), and Dox (1910).

It was thought advisable to repeat the study of the influence of reaction upon the enzyme activities of microörganisms. One per cent peptone solutions were made up neutral to phenolphthalein, and divided into three portions; one portion was left at that reaction and is termed "alkaline;" to a second portion 3 cc. of 0.1N HCl were added for each gram of casein or peptone,

this solution being termed "neutral;" to the third portion 5 cc. of 0.1N HCl were added for each gram of casein and peptone, this solution being termed "acid." *A. niger* and *A. ochraceus* grown for fourteen days on Czapek's and Peptone-Czapek solutions were used. The enzyme cultures were incubated for four days.

TABLE 3

*The influence of composition of medium and reaction upon the production of amino nitrogen from proteins by the exoenzymes of microörganisms*

| ORGANISMS USED | CULTURE MEDIUM | AGE OF CULTURE | MILLIGRAMS OF $NH_3$-N PER 100 CC. OF SOLUTION | | | |
|---|---|---|---|---|---|---|
| | | | 1 per cent peptone | | 1 per cent casein | |
| | | | Phenolphthalein* | Litmus* | Phenolphthalein | Litmus |
| | | *days* | | | | |
| Control | | | 21.24 | 21.24 | 7.26 | 7.26 |
| *A. niger* | Czapek's | 5 | 21.46 | 34.16 | 23.78 | 26.10 |
| *A. niger* | Czapek's | 14 | 21.30 | 22.20 | 38.88 | 19.44 |
| *A. ochraceus* | Czapek's | 5 | 21.88 | 33.64 | 30.60 | 34.80 |
| *A. ochraceus* | Czapek's | 14 | 22.62 | 23.78 | 9.86 | 16.82 |
| *A. ochraceus* | Peptone-Czapek | 14 | 39.44 | 45.24 | 41.76 | 50.76 |
| *A. fuscus* | Czapek's | 14 | 21.40 | 23.56 | 20.30 | 7.54 |
| *A. fuscus* | Peptone-Czapek | 14 | 41.56 | 50.76 | | |
| *P. chrysogenum* | Peptone-Czapek | 8 | 28.12 | 62.48 | 19.71 | 19.71 |
| *Citr. glaber* | Czapek's | 5 | 39.44 | 33.06 | 31.90 | 39.44 |
| *Act. californicus* | Czapek's | 15 | 33.06 | 47.56 | 31.32 | 52.20 |
| *Act. sp.* 101 | Czapek's | 15 | 23.78 | 27.04 | 7.54 | 7.40 |
| *Act. sp.* 101 | Peptone-Czapek | 15 | 33.06 | 30.16 | 45.82 | 45.24 |
| *Act. sp.* 101 | Peptone-Czapek | 38 | 70.38 | 52.17 | 75.02 | 53.50 |
| *Act. violaceus-ruber* | Czapek's | 90 | 28.71 | 28.71 | 17.25 | 23.64 |
| Trypsin: 200 mgm. | | | 60.90 | 45.24 | 81.20 | 56.26 |
| Pepsin: 200 mgm. | | | 22.62 | 26.68 | 13.92 | 39.44 |

* Phenolphthalein = neutral to phenolphthalein, litmus = neutral to litmus.

The data presented in table 4 confirm the previous observations concerning the nature of the exoenzymes produced by microörganisms. In this case also the peptone containing media contained a more active enzyme or a stronger concentration of proteolytic enzymes than the non-protein media. As to the

influence of reaction, the highest production of amino nitrogen took place, in most instances, when the reaction was almost neutral to litmus. All the three reactions proved to be more or less favorable to the enzyme action, but the medium reaction which was not too acid nor too alkaline gave the best results. The enzymes of the fungi studied seem to have a wider range of reaction than the animal enzymes.

To obtain further information on the influence of the reaction upon the activities of the enzymes studied and also to throw some light upon the possibility of their being a mixture of two

TABLE 4

*Influence of reaction upon the enzyme activities of microörganisms*

| ORGANISMS USED | CULTURE MEDIUM | REACTION OF SOLUTION | MILLIGRAMS OF $NH_3$-N PER 100 CC. OF SOLUTION | |
|---|---|---|---|---|
| | | | 1 per cent peptone | 1 per cent casein |
| Control | | | 21.40 | 7.28 |
| *A. niger* | Czapek's | Alkaline | 23.00 | 12.40 |
| *A. niger* | Czapek's | Neutral | 23.60 | 25.60 |
| *A. niger* | Czapek's | Acid | 21.44 | 13.44 |
| *A. niger* | Peptone-Czapek | Alkaline | 37.18 | 19.44 |
| *A. niger* | Peptone-Czapek | Neutral | 64.90 | 39.56 |
| *A. niger* | Peptone-Czapek | Acid | 25.00 | 24.80 |
| *A. ochraceus* | Czapek's | Alkaline | 24.92 | 53.34 |
| *A. ochraceus* | Czapek's | Neutral | 30.70 | 38.96 |
| *A. ochraceus* | Czapek's | Acid | 21.26 | 27.36 |
| *A. ochraceus* | Peptone-Czapek | Alkaline | 44.88 | 69.32 |
| *A. ochraceus* | Peptone-Czapek | Neutral | 55.14 | 73.76 |
| *A. ochraceus* | Peptone-Czapek | Acid | 32.50 | 74.84 |

kinds of enzymes, one acting best in a slightly acid and the other in an alkaline reaction, the following experiment was made. Exo- and endoenzymes of *A. niger* were obtained by the previously described methods from eight-day-old cultures grown on the Peptone-Czapek solution. Twenty cubic centimeters of the filtrate containing the exoenzyme and 800 mgm. of the mycelium containing the endoenzyme were added to 1 per cent peptone and casein solutions. The reaction of the substrata was made neutral to litmus, which was found in the previous experiments to be the best reaction for the activity of these enzymes. The

substrata plus enzymes were incubated for forty-eight hours at 37°C. At the end of that period, the amino nitrogen content of the cultures was determined; one set of cultures was then made neutral to phenolphthalein (no. 2 in table 5), while the others were left untouched (no. 1 in the table); the cultures were then further incubated for forty-eight hours and amino nitrogen determined. The results are given in table 5.

It is apparent that, if the proteolytic enzymes of *A. niger* are a mixture of several enzymes, the possibility of the existence of one enzyme which may act in a reaction optimum for tryptic activities is excluded, unless that enzyme was destroyed while the

TABLE 5

*Influence of reaction upon the enzyme activities of A. niger.*

| REACTION | PERIOD OF INCUBATION | MILLIGRAMS OF $NH_2$=N PER 100 CC. OF SOLUTION | | | |
|---|---|---|---|---|---|
| | | 1 per cent peptone | | 1 per cent casein | |
| | | Exoenzyme | Endoenzyme | Exoenzyme | Endoenzyme |
| | *hours* | | | | |
| Control | | 21.36 | 20.24 | 7.24 | 6.30 |
| 1. Neutral to litmus | 48 | 30.80 | 50.40 | 16.80 | 39.20 |
| 1. Neutral to litmus | 96 | 31.08 | 56.00 | 17.92 | 56.40 |
| 2. Neutral to phenolphthalein | 48 | 30.64 | 50.54 | 16.52 | 38.46 |
| 2. Neutral to phenolphthalein | 96 | 30.08 | 49.92 | 15.40 | 37.58 |

culture was incubated for the first forty-eight hours; which is very doubtful, since the medium itself, where the enzyme is produced, is very acid. The H-ion concentration of the filtrate of the eight-day-old culture of *A. niger* grown on the Peptone-Czapek medium was often as high as pH = 2.0. The fact is that the neutralization of the substratum to phenolphthalein, after forty-eight hours of incubation at a neutral reaction, checked the further activity of the enzyme, as is seen in table 5, where the amount of amino nitrogen is found to increase in the neutral culture, while the culture made alkaline gave no further increase; the slight decrease found is due to the dilution of the substratum through the addition of the alkali for change of re-

action. Long and Hull (1917) have recently shown that trypsin acts on fibrin and fibrin-peptone most energetically at an H-ion concentration between $10^{-8}$ and $5.10^{-9}$, and on casein at $3.10^{-6}$ to $5.10^{-7}$. The enzyme of *A. niger*, although acting on casein at a reaction similar to the one found to be optimum for trypsin

TABLE 6

*Influence of age of culture upon the activities of proteolytic exo- and endoenzymes of microörganisms*

| ORGANISMS USED | CULTURE MEDIUM | INCUBATION | MILLIGRAMS OF $NH_2$-N PER 100 CC. OF SOLUTION | | | |
|---|---|---|---|---|---|---|
| | | | 1 per cent peptone | | 1 per cent casein | |
| | | | Exoenzyme | Endoenzyme | Exoenzyme | Endoenzyme |
| | | *days* | | | | |
| Control | | | 21.24 | 20.35 | 7.18 | 6.22 |
| *A. niger* | Czapek's | 3 | 26.30 | 53.41 | 12.27 | 90.60 |
| *A. niger* | Czapek's | 10 | 21.64 | 36.42 | 11.38 | 33.00 |
| *A. niger* | Czapek's | 18 | 21.84 | 39.76 | 10.64 | 28.00 |
| *A. niger* | Czapek's | 35 | 21.76 | 34.77 | 7.84 | 15.39 |
| *A. niger* | Peptone-Czapek | 3 | 28.67 | 84.82 | 19.89 | 125.77 |
| *A. niger* | Peptone-Czapek | 10 | 24.26 | 59.75 | 25.71 | 80.80 |
| *A. niger* | Peptone-Czapek | 18 | 29.12 | 64.40 | 21.28 | 92.40 |
| *A. niger* | Peptone-Czapek | 35 | 46.74 | 43.89 | 18.24 | 22.33 |
| *A. ochraceus* | Czapek's | 3 | 26.32 | 47.97 | 19.31 | 38.02 |
| *A. ochraceus* | Czapek's | 10 | 31.86 | 68.28 | 43.87 | 73.97 |
| *A. ochraceus* | Czapek's | 18 | 34.72 | 71.68 | 50.40 | 82.88 |
| *A. ochraceus* | Czapek's | 35 | 22.46 | 57.00 | 20.32 | 76.95 |
| *A. ochraceus* | Peptone-Czapek | 3 | 26.91 | 49.14 | 53.24 | 55.58 |
| *A. ochraceus* | Peptone-Czapek | 10 | 62.59 | 80.80 | 58.03 | 79.66 |
| *A. ochraceus* | Peptone-Czapek | 18 | 42.56 | 93.52 | 64.40 | 115.92 |
| *A. ochraceus* | Peptone-Czapek | 22 | 62.70 | 64.26 | 68.40 | 74.10 |

by Long and Hull, acts on peptone and on fibrin (as will be seen later) at a higher H-ion concentration.

To throw more light upon the influence of the age of the culture on the proteolytic activities of both kinds of enzymes, it was thought advisable to grow *A. niger* and *A. ochraceus* on both media, in a large number of flasks. At the end of the proper incubation period, single flasks containing each organism were taken out of the incubator and filtered. The mycelium was

treated by the acetone method, and 0.8 gram of the dried mycelium was used in each case. The organisms were grown for 3, 10, 18, 35 days at 28°C.; the enzyme cultures were in all cases incubated for four days at 37°C. The amino nitrogen produced due to the activities of the enzymes was determined as usual, and results calculated back to 100 cc. of the substrata.

When we compare the influence of the medium upon the activities of the enzymes, we see clearly that the presence of peptone in the medium greatly increases the activities of the enzymes, both extra- and intracellular, in their action on peptone and casein, as shown in table 7; this effect is observed with all the periods of incubation studied. It is remarkable that in most cases *A. niger* produced the strongest enzymes in the first period of incubation, namely in three days, particularly when grown on the peptone free medium. There is a more or less steady decrease in the activities of the enzymes with the increase of the incubation period, and the enzymes obtained from the organism grown on the peptone medium particularly the exoenzymes, show greater irregularities in this respect.

In the case of the enzyme obtained from *A. ochraceus*, we find that the activities of the enzymes increased from the third till the eighteenth day of incubation of the organism, further incubation giving a decrease in the activities of the enzymes. Only in two cases is an increase in the activities of the enzymes found after the growth of the organism was continued for a period greater than eighteen days, namely in the case of the exoenzyme of that organism growing on peptone. The difference in the activities of the two organisms may be explained on the following assumptions: *A. niger* grows very rapidly and produces a great deal of acid, mostly oxalic and citric, in both media; while *A. ochraceus* grows comparatively slowly and produces no acid at all or very little. The rapid growth of *A. niger* will result in an early production of strongly acting enzymes; the acids produced by this organism may act injuriously upon the enzymes and their activity will therefore be strongest in the earliest period of incubation. The slower growth of *A. ochraceus* will result in a lack of strongly acting enzymes in the early stages of the growth

of the organism, but their activity will increase with the age of the organism, since no acids or other harmful agents are produced, till the organism begins to autolyze, and then the further production of the enzymes may be affected by the products of

TABLE 7

*Influence of incubation of enzymes upon the amino nitrogen produced*

| ORGANISMS USED | CULTURE MEDIUM | AGE OF CULTURE | INCUBATION | MILLIGRAMS OF $NH_2$=N PER 100 CC. OF SOLUTION | | | |
|---|---|---|---|---|---|---|---|
| | | | | 1 per cent peptone | | 1 per cent casein | |
| | | | | Exoenzyme | Endoenzyme | Exoenzyme | Endoenzyme |
| | | *days* | *hours* | | | | |
| Control | | | | 21.46 | 20.30 | 7.30 | 6.28 |
| *A. niger* | Peptone-Czapek | 10 | 2 | | 24.34 | | 15.85 |
| *A. niger* | Peptone-Czapek | 10 | 4 | | 32.26 | | 21.51 |
| *A. niger* | Peptone-Czapek | 10 | 18 | | 42.81 | | 34.47 |
| *A. niger* | Peptone-Czapek | 10 | 23 | | 46.90 | | 43.51 |
| *A. niger* | Peptone-Czapek | 10 | 40 | | 52.63 | | 47.09 |
| *A. niger* | Peptone-Czapek | 10 | 88 | | 54.90 | | 50.51 |
| *A. niger* | Peptone-Czapek | 10 | 112 | | 61.94 | | 58.07 |
| *A. niger* | Peptone-Czapek | 10 | 160 | | 63.60 | | |
| *A. niger* | Peptone-Czapek | 18 | 2 | | 28.30 | | 17.55 |
| *A. niger* | Peptone-Czapek | 18 | 4 | | 29.43 | | 22.64 |
| *A. niger* | Peptone-Czapek | 18 | 18 | | 40.03 | | 44.48 |
| *A. niger* | Peptone-Czapek | 18 | 23 | | 42.38 | | 50.98 |
| *A. niger* | Peptone-Czapek | 18 | 40 | | 52.08 | | 59.28 |
| *A. niger* | Peptone-Czapek | 18 | 88 | | 59.72 | | 74.10 |
| *A. niger* | Peptone-Czapek | 18 | 112 | | 61.94 | | 76.31 |
| *A. ochraceus* | Peptone-Czapek | 10 | 2 | 31.13 | 30.00 | 32.26 | 30.55 |
| *A. ochraceus* | Peptone-Czapek | 10 | 4 | 33.96 | 33.39 | 33.96 | 35.66 |
| *A. ochraceus* | Peptone-Czapek | 10 | 18 | 41.14 | 41.14 | 46.15 | 57.88 |
| *A. ochraceus* | Peptone-Czapek | 10 | 23 | 43.51 | 45.20 | 51.98 | 74.02 |
| *A. ochraceus* | Peptone-Czapek | 10 | 40 | 47.64 | 51.52 | 60.94 | 84.76 |
| *A. ochraceus* | Peptone-Czapek | 10 | 88 | | 53.80 | 62.04 | |
| *A. ochraceus* | Peptone-Czapek | 10 | 112 | | 56.96 | 63.60 | |
| *A. ochraceus* | Peptone-Czapek | 10 | 160 | | 67.56 | 70.38 | |

autolysis. The difference in behavior of the enzymes of *A. niger* grown on the peptone medium may be explained by the fact that the peptone and its decomposition products present in the medium may exert a buffer action upon the acids of the medium thus decreasing their injurious effect.

The following experiment was made with the purpose of demonstrating the influence of the incubation of the enzyme cultures upon the amino nitrogen production, the reaction of the substrata being neutral to litmus.

When the velocities of reaction of the different enzymes are compared, one finds in all cases an initial rapid increase in the amounts of amino nitrogen produced, followed by a gradual decrease in velocity.

The enzyme cultures were incubated in all previous experiments at 37°C. which was found to be the optimum temperature for the action of the proteolytic enzymes derived from animal tissues. But since the action of the enzymes of microörganisms differs so much from that of the animal enzymes, it was thought advisable to study the influence of incubation upon the activities of the enzymes used in this work. Both exo- and endoenzymes of *A. niger* grown for eight days on the Peptone-Czapek medium were used. Twenty cubic centimeters of the exoenzyme containing filtrate and 0.8 gram of the endoenzyme containing mycelium were added to peptone and casein solutions so as to make the concentration of the substrata just 1 per cent; the reaction was made neutral to litmus and the proper disinfectants were added. The cultures were incubated at 12°, 23°, 29°, 34°, and 39°C. for forty-eight hours; at the end of that period the amino nitrogen was determined in all the solutions.

The facts brought out in table 8 tend to show that the range of temperature optimum for the action of the enzymes of *A. niger* is comparatively great. Even at as low a temperature as 12°C. the splitting of the proteins still took place, the action increasing with the increase in temperature. The optimum was reached at 29° to 34°C., followed by a drop in activity at 39°C. We would naturally expect that an organism, whose temperature optimum lies below the optimum for the enzymes of the warm blooded animals, should produce enzymes which will act best at somewhat lower temperatures.

The question as to the nature of the enzymes of microörganisms cannot be definitely answered as yet from the previous experiments. It has been proven that both exo- and endo-

enzymes of the microörganisms used can decompose peptone and casein, but this does not tell us with what group of enzymes these should be classified, whether with the trypsins or with the erepsins, both of which, when obtained from animals, split peptone and casein. But these are active only in alkaline solutions, while the enzymes of the microörganisms studied act best in neutral and even slightly acid media. This would seem to indicate that the latter do not belong to the same class of enzymes as those obtained from animal tissues. Went (1901), Butkewitch (1903), Saito (1903), and Franceschelli (1915) claimed that we are dealing with trypsins. The work of Dox (1910)

TABLE 8

*Influence of temperature of incubation upon the activities of the proteolytic enzymes of A. niger*

| TEMPERATURE | MILLIGRAMS OF $NH_2$=N PER 100 CC. OF SOLUTION | | | |
|---|---|---|---|---|
| | 1 per cent peptone | | 1 per cent casein | |
| | Exoenzyme | Endoenzyme | Exoenzyme | Endoenzyme |
| *deg. C.* | | | | |
| Control | 21.46 | 20.40 | 7.24 | 6.18 |
| 12 | 29.18 | 33.60 | 11.24 | 11.80 |
| 23 | 29.46 | 48.72 | 14.00 | 20.72 |
| 29 | 33.04 | 56.00 | 16.80 | 56.28 |
| 34 | 34.16 | 69.44 | 21.12 | 56.00 |
| 39 | 30.24 | 45.36 | 17.36 | 39.20 |

Reed and Stahl (1911), Berman and Rettger (1916) and others would lead us to think that we are dealing here with erepsins, which may be very close to animal erepsin. The work of Vines (1900–1910) on the enzymes of plants and those of *Aspergillus oryzae* and the studies of Hagem (1910) tend to prove that we have here a mixture of peptases and ereptases more closely allied to the corresponding plant enzymes than to the animal enzymes. The fact that certain investigators have obtained the liquefaction of gelatin, which resulted in the production of peptone, by the enzyme of *A. niger* leads us to think that this organism produces enzymes which, besides being of an ereptic nature, are also tryptic or peptic in nature. The work of Malfitano (1900) and

of Steffens (1900) tend to show that the enzymes of molds are entirely different from animal enzymes.

Erepsin, as was shown by Cohnheim (1901), Fränkel (1916) and other investigators, decomposes not only peptone but also casein, so that the two substances used in the previous experiments cannot give us any differentiation between erepsin and other enzymes which could also decompose true proteins. Merck's fibrin and crystalline egg-albumen were therefore used

TABLE 9

*Decomposition of fibrin and crystalline egg-albumen by enzymes of microörganisms*

| ORGANISMS USED | CULTURE MEDIUM | AGE OF CULTURE | MILLIGRAMS OF $NH_2$=N PER 100 CC. OF SOLUTION | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1 per cent fibrin | | | 1 per cent egg-albumen* | | |
| | | | Exoenzyme | | Endoenzyme | Exoenzyme* | | Endoenzyme* |
| | | | Control | Enzyme | Control=0 | Control | Enzyme | Control = 7.4 |
| | | *days* | | | | | | |
| *A. niger* | Czapek's | 35 | 1.28 | 19.92 | 21.05 | 8.02 | 18.21 | 22.76 |
| *A. niger* | Peptone-Czapek | 18 | | | 22.76 | | | 46.66 |
| *A. niger* | Peptone-Czapek | 35 | 11.10 | 21.05 | 6.26 | 13.16 | 27.31 | 25.61 |
| *A. ochraceus* | Czapek's | 10 | 1.14 | 46.66 | 14.23 | 8.16 | 29.59 | 34.14 |
| *A. ochraceus* | Czapek's | 35 | 1.64 | 11.99 | 25.04 | 8.28 | 11.38 | 44.38 |
| *A. ochraceus* | Peptone-Czapek | 10 | 10.04 | 40.40 | 82.51 | 12.48 | 32.43 | 38.69 |
| *A. ochraceus* | Peptone-Czapek | 35 | 12.16 | 27.31 | 31.30 | 14.26 | 40.40 | 30.16 |
| *P. chrysogenum* | Czapek's | 35 | 2.56 | 52.92 | | 8.64 | 34.71 | |
| *Act. griseus* | Czapek's | 90 | 0 | 36.42 | | 7.96 | 23.33 | |

* The presence of amino nitrogen in the crystalline egg-albumen may be due either to the end group of the protein molecule, or to such product combined with a small quantity of ammonia, remaining as a contamination of the protein in the form of $(NH_4)_2SO_4$.

for the next experiment. The fibrin was introduced, in small pieces into flasks containing 100 cc. of water so as to make one per cent of the liquid, after the proper enzyme has been added. The crystalline egg-albumen was also made up as a 1 per cent solution in water. The exo- and endoenzymes of *A. niger*, *A. ochraceus*, *P. chrysogenum*, and *Act. griseus* were used for this experiment.

As is seen from table 9, such true proteins as fibrin and crystalline egg-albumen are also decomposed by the enzymes of the

microörganisms studied; the different enzymes behaved somewhat differently in this case, the largest amount of amino nitrogen being obtained from fibrin by the exoenzymes of *P. chrysogenum* and by the endoenzymes of *A. ochraceus*.

The digestion of fibrin and crystalline egg-albumen is shown to be positive, hence the enzymes of the microörganisms cannot be erepsins or only ereptic in nature; although it is possible that other organisms behave in an entirely different way from those studied in this paper.

It still remained to find out whether the enzymes are tryptic in nature. Otsuka (1916) claimed that trypsin retains its activity after filtration through a Chamberland filter, but erepsin becomes inactive. The filtrate of a culture of *A. niger* grown for eight days on the Peptone-Czapek solution was filtered through a no. 6 Pasteur-Chamberland filter, and both filtered and unfiltered portions were compared with the untreated liquid as to their enzymatic power. The tests were performed in the ordinary way, by adding 20 cc. of each fluid to 80 cc. of peptone and casein solutions, so as to make them of 1 per cent concentration; these were incubated for forty-eight hours at 37°C. and amino nitrogen determined.

As is seen from table 10, the filtering of the enzyme culture through a porcelain filter results only in a very slight diminution in the activity of the enzymes, which may be due to a mere mechanical retention by certain colloidal particles of a small quantity of the enzyme. If the results of Otsaka hold true for erepsin, the enzymes of the microörganisms studied do not seem to be erepsins.

Robertson (1907) pointed out the fact that when a saturated solution of safranin is added to a neutral or faintly alkaline solution of trypsin, a flocculent precipitate is formed which contains the most active constituents of the trypsin. When this precipitate is added to a solution of a protein, the latter will be decomposed very rapidly, while the filtrate left, after the safranin-trypsin precipitate is removed, is almost inactive. This behavior of the safranin toward the trypsin in solution can be utilized for the testing of the tryptic nature of the exoenzymes of

microörganisms. Two liters of the filtrate from an eight-day-old culture of *A. niger* grown on the Peptone-Czapek solution were used for this work; this filtrate was strongly acid due to the large quantities of oxalic and citric acid produced by the organism. One liter of the fluid was left acid and the other was neutralized to phenolphthalein by means of N NaOH. To each liter of the fluid 20 cc. of a saturated solution of safranin were added. A flocculent colored precipitate appeared in both instances, but was much heavier in the neutral solution. The precipitates were allowed to settle for twenty-four hours, then filtered off, washed with alcohol, and dried over sulfuric acid. The precipitate from the liter of medium left acid weighed 60 mgm. and that from the liter of neutral medium weighed 500

TABLE 10

*Influence of filtration through a porcelain filter upon the action of exoenzymes of A. niger*

| | Milligrams of $NH_2$=N per 100 cc. of solution | | | | | |
|---|---|---|---|---|---|---|
| | 1 per cent peptone | | | 1 per cent casein | | |
| | Untreated culture | Filtered portion | Unfiltered portion | Untreated culture | Filtered portion | Unfiltered portion |
| At start | 21.38 | 21.30 | 21.36 | 7.44 | 7.24 | 7.42 |
| After 48 hours | 28.62 | 25.16 | 27.32 | 15.77 | 12.30 | 13.88 |

mgm. The precipitates and the filtrates left, after their removal were now tested for their proteolytic activities. About one-half of each precipitate and 20 cc. of the filtrate left were used as the sources of enzymes; as substrata 100 cc. of 1 per cent peptone and casein solutions were used. The cultures were incubated for forty-eight hours at 37°C.

It is seen from table 11 that the safranin does not precipitate the enzyme from the culture medium. One liter of medium, or just 50 times as much as is ordinarily used for the inoculation of 100 cc. of the 1 per cent substratum, did not give, on precipitation with safranin, enough enzyme to decompose as much of the protein, as 20 cc. of the solution usually does. We might therefore conclude that the enzyme is not of the nature of trypsin.

The small quantity of substratum decomposed by the safranin precipitate might be merely due to the fact that some of the enzyme was carried down mechanically by the safranin precipitate, and not in the form of a salt such as the color-base safranin is supposed to form with the acid-like trypsin. The filtrates from the safranin precipitate were weaker in their enzymatic action than the normal solution obtained by filtering the original culture of the organism. This may be due to several reasons: first, the filtrate from the safranin precipitate was slightly diluted by the addition of the safranin solution; second, some of the enzyme might have been carried down by the precipitate; and third, the safranin itself may have had some injurious effect upon the enzyme. There does not seem to be any doubt that

TABLE 11

*The action of safranin as a precipitating agent for the ezoenzymes of A.niger*

| | MILLIGRAMS OF $NH_3$=N PER 100 CC. OF SOLUTION | | | | | |
|---|---|---|---|---|---|---|
| | 1 per cent peptone | | | 1 per cent casein | | |
| | Normal exoenzyme | Precipitate | Filtrate | Normal exoenzyme | Precipitate | Filtrate |
| Control | 21.40 | 20.24 | 21.12 | 7.36 | 6.12 | 7.20 |
| Acid | | 22.96 | 28.40 | | 6.72 | 11.76 |
| Neutral | 34.16 | 31.92 | 29.12 | 29.68 | 8.96 | 12.88 |

the enzymes of many microörganisms can attack true proteins. What, then, is the true nature of the enzymes? They cannot be similar to the animal trypsins, because they act best in a neutral and even slightly acid medium, are not precipitated by safranin, and act best at lower temperatures. They cannot be pepsins alone, because they decompose peptones and the splitting of casein and other proteins goes further than the peptone stage. The only thing that could be suggested is that these enzymes should not be classified with the animal proteolytic enzymes at all. If anything, they approach nearer the plant enzymes and they are either tryptases similar to the animal trypsins, but differentiated from them by certain characteristics; or they are a

mixture of peptases and ereptases, as Vines (1900–1910) suggested for plants.

Corper and Sweany (1917) claimed that the tubercle bacilli possess a tryptic-like enzyme capable of splitting proteins in alkaline solutions, a weak pepsin-like enzyme capable of splitting proteins in acid solutions, and an erepsin-like enzyme capable of decomposing peptones in acid solutions. As a matter of fact, the same enzymatic activities are found to hold true also for the molds and the actinomycetes studied, and the explanation of the co-existence of the three different enzymes in the same organism can be used here also as an explanation of the peculiar behavior of the enzymes of the microörganisms studied. This would simplify greatly the question as to the nature of the enzymes of microörganisms, but the explanation would be of doubtful importance in explaining the true nature of the enzymes of the organisms studied. Before we are able actually to separate the different enzymes, the only assumption that can be made is that the proteolytic enzymes of microörganisms behave in a different manner from animal enzymes and should be placed in a class by themselves.

## SUMMARY

1. The proteolytic enzymes contained in the fungi studied appear to differ from known proteolytic enzymes of animal origin in the following particulars:

*a.* Their range of optimum reaction is greater; a reaction neutral to litmus was found to be the optimum one for the activities of the majority of the enzymes studied.

*b.* The temperature optimum is somewhat lower.

*c.* Although acting best in a neutral or sometimes in a slightly acid medium they differ from animal trypsin in not being precipitated by safranin.

*d.* The exoenzymes can pass through a Pasteur-Chamberland filter.

2. The sugar content of the medium has no influence upon the production of proteolytic exo- and endoenzymes of *A. niger*.

3. Both exo- and endoenzymes are produced by microörganisms on protein-containing and protein-free media, but the activities of the enzymes from organisms grown on the media containing proteins are greater than those obtained from organisms grown on the protein-free media.

4. The age of the culture, at which the most active enzymes are obtained, depends on the organism itself, its rapidity of growth, and the nature of the waste products produced in the medium.

5. Fibrin and crystalline egg-albumen are decomposed by both the exo- and endoenzymes of the organisms used.

6. Small quantities of ammonia were found to be produced in the decomposition of peptone and casein by the proteolytic enzymes of the microörganisms studied. This fact indicates the probable presence of desamidases among the enzymes produced.

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# SOME OBSERVATIONS ON THE EFFICIENCY OF THE PRESENT STANDARD AGAR FOR THE ESTIMATION OF BACTERIA IN MILK

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On receipt of the last report of the Committee on Standard Methods[1] the workers in this laboratory noted with surprise and also with some skepticism that the content of nutrient substances in the standard agar had been reduced still further. We accepted the revision of methods, however, in their entirety and have been adhering to the new standards ever since but with an attitude somewhat more critical than heretofore. Whether it was due to this critical attitude or whether it was on account of the change in the composition of the standard agar, it is a fact that we soon noticed inaccuracies and inconsistencies in some of our results which were too important to be overlooked.

The chief difficulty which we have encountered, and for which we are constrained to blame the present standard agar, consists in the failure of the counts made from the different dilutions of the same sample to check with each other. It was not infrequently noticed, for example, that the plates from the 1–100 dilution would give the sample a total count many times greater than those from the 1–1000 dilution. Very often it would be quite impossible to count the former while the latter would show fewer than the minimum of 20 colonies. This sort of a situation could only leave the worker the choice of estimating the number of colonies on the very much overcrowded 1–100 plates or of reporting the number indicated by the obviously unreliable 1–1000 plates.

[1] Provisional Report of the Committee on Standard Methods of Bacteriological Analysis of Milk, 1916, Am. Jour. Pub. Health, **6**, 1315.

It was always observed that when the experience just described occurred it would be found that the 1–100 plates contained large numbers of very small round colonies resembling streptococcus colonies but differing from the latter in being whiter and more opaque. These tiny colonies ranged in size from that of a pin-head, to the merest points. The recognition of the latter as colonies, even with the aid of the standard lens, was practically impossible. It was only the gradation in size that indicated that they were colonies and not specks in the medium. These very minute colonies appeared on the 1–1000 plates only very

TABLE 1

| TOTAL BACTERIA PER CUBIC CENTIMETER INDICATED BY 1-100 COUNTS | TOTAL BACTERIA PER CUBIC CENTIMETER INDICATED BY 1-1000 COUNTS |
|---|---|
| 44,000 | 16,000 |
| 15,000 | 5,000 |
| 39,000 | 4,000 |
| 74,000 | 28,000 |
| 46,000 | 3,500 |
| 19,000 | 7,000 |
| 90,000 | 1,000 |
| 28,000 | 9,000 |
| 18,000 | 3,000 |
| 20,000 | 8,500 |
| 11,000 | 1,000 |
| 16,000 | 1,500 |
| 43,000 | 10,000 |
| 29,000 | 8,500 |

rarely, and when they did they often were found on only one of the duplicates. Their appearance always accompanied a wide difference in the counts of the duplicate plates. Table 1 gives the counts of fourteen samples selected from our records.

The inconsistencies described seemed very mysterious at first but on consideration it was seen that the explanation must lie in the difference in composition between the nutrient medium in the 1–100 plates and that in the 1–1000 plates. Inasmuch as the same nutrient agar was used in each this difference could only arise from the amount of milk introduced in the sample. One one hundredth cubic centimeter of milk introduced into a

10 cc. quantity of the standard agar so enriched the latter that certain organisms could multiply to the extent of producing visible colonies while these organisms could not do so when only 0.001 cc. of milk was added. In other words, the addition to, or elimination from, a tube of agar of 0.009 cc. of milk was sufficient to change very materially the growth supporting qualities of the medium. It appeared then that to keep the medium of constant composition in all dilutions it was only necessary to supply to the 1–1000 dilution and all higher dilutions enough milk to make up the deficiency. Ths was done approximately by making the higher dilutions in sterile 1–100 milk instead of in sterile water. Table 2 gives the results of a preliminary test to determine the efficiency of this improvement. In this initial test the 1–100 dilution was plated out in duplicate, but only one plate was made from each of the two 1–1000 dilutions. In a second test, the results of which are given in table 3, all three dilutions were plated out in duplicate. For convenience the columns showing the counts on the 1–100 plates are marked "C" and those showing the counts on the plates made from the 1–1000 dilutions in sterile water and sterile 1–100 milk are marked "$M_1$" and "$M_2$" respectively. The samples employed in these experiments were not selected samples. They were picked at random from those brought in for examination.

Several important points are noticed on examination of the figures in tables 2 and 3. In the first place it is observed that in many samples the $M_2$ count is considerably higher than the $M_1$ count and that the $M_2$ counts check better with the C counts than do the $M_1$ figures. This establishes the validity of the reasoning which was the basis of the experiment. The added milk enhances the growth supporting qualities of the medium. It is interesting also to note that in the majority of cases in which these differences appear the samples are those of pasteurized milk. In going through our records to get the figures given in table 1, samples were selected which best presented the inconsistencies mentioned. It was not noticed until afterwards that every sample was one of pasteurized milk.

Another fact, which is not indicated in the tables, but which is very apparent to the person doing the counting, is that in cases in which the very small colonies appear on both the $M_1$

TABLE 2

| SAMPLE NUMBER | GRADE | TOTAL NUMBER OF BACTERIA PER CUBIC CENTIMETER INDICATED BY THE DIFFERENT PLATE COUNTS | | |
|---|---|---|---|---|
| | | C | $M_1$ | $M_2$ |
| 1 | Raw | 3,300 | 0 | 2,000 |
| 2 | Raw | 14,000 | 4,000 | 14,000 |
| 3 | Raw | 90,000 | 130,000 | 110,000 |
| 4 | Raw | 2,600 | 2,000 | 5,000 |
| 5 | Raw | 42,000 | 22,000 | 29,000 |
| 6 | Raw | 1,500 | 0 | 1,000 |
| 7 | Raw | 2,500 | 0 | 3,000 |
| 8 | Raw | 3,400 | 7,000 | 3,000 |
| 9 | Raw | 1,400 | 0 | 1,000 |
| 10 | Raw | 5,600 | 5,000 | 9,000 |
| 11 | Raw | 86,000 | 140,000 | 150,000 |
| 12 | Raw | 200,000 | 150,000 | 490,000 |
| 13 | Raw | | 150,000 | 210,000 |
| 14 | Raw | | 140,000 | 260,000 |
| 15 | Raw | 17,000 | 13,000 | 36,000 |
| 16 | Raw | 9,400 | 14,000 | 17,000 |
| 17 | Raw | 38,000 | 27,000 | 34,000 |
| 18 | Raw | 8,600 | 8,000 | 5,000 |
| 19 | Raw | 12,000 | 16,000 | 10,000 |
| 20 | Raw | 66,000 | 70,000 | 72,000 |
| 21 | Raw | 95,000 | 95,000 | 120,000 |
| 22 | Pasteurized | 16,000 | 7,000 | 18,000 |
| 23 | Pasteurized | 9,700 | 4,000 | 10,000 |
| 24 | Pasteurized | 40,000 | 8,000 | 40,000 |
| 25 | Pasteurized | 43,000 | 9,000 | 37,000 |
| 26 | Pasteurized | 250,000 | 9,000 | 400,000 |
| 27 | Pasteurized | 250,000 | 0 | 9,000 |
| 28 | Pasteurized | 200,000 | 8,000 | 370,000 |
| 29 | Pasteurized | 26,000 | 0 | 39,000 |
| 30 | Pasteurized | 24,000 | 0 | 18,000 |
| 31 | Pasteurized | 12,000 | 3,000 | 8,000 |

and $M_2$ plates they are very much larger and more easily counted on the latter.

A qualitative examination of the minute colonies which were the source of the errors described above revealed the fact that

they were all composed of organisms of the lactic acid type. A large number were isolated and all were found to coagulate milk rapidly and to acidify glucose and lactose broths without the production of gas. Most of them were Gram negative bacilli, but diplococcus types were isolated from several samples.

TABLE 3

| SAMPLE NUMBER | GRADE | TOTAL NUMBER OF BACTERIA PER CUBIC CENTIMETER INDICATED BY THE DIFFERENT PLATE COUNTS | | |
|---|---|---|---|---|
| | | C | $M_1$ | $M_2$ |
| 1 | Raw | 26,000 | 31,000 | 56,000 |
| 2 | Raw | 25,000 | 10,000 | 28,000 |
| 3 | Raw | 47,000 | 58,000 | 57,000 |
| 4 | Raw | 28,000 | 32,000 | 31,000 |
| 5 | Raw | 27,000 | 23,000 | 33,000 |
| 6 | Raw | 7,500 | 8,000 | 7,000 |
| 7 | Raw | 21,000 | 18,000 | 30,000 |
| 8 | Raw | 2,700 | 6,500 | 5,000 |
| 9 | Raw | 8,200 | 8,000 | 8,500 |
| 10 | Raw | 5,600 | 6,000 | 9,500 |
| 11 | Raw | 45,000 | 36,000 | 43,000 |
| 12 | Raw | 7,300 | 10,000 | 11,000 |
| 13 | Raw | 30,000 | 54,000 | 38,000 |
| 14 | Raw | 3,400 | 3,500 | 4,500 |
| 15 | Raw | 11,000 | 16,000 | 15,000 |
| 16 | Pasteurized | 25,000 | 4,500 | 32,000 |
| 17 | Pasteurized | 120,000 | 46,000 | 160,000 |
| 18 | Pasteurized | 73,000 | 60,000 | 80,000 |
| 19 | Pasteurized | 73,000 | 110,000 | 160,000 |
| 20 | Pasteurized | 19,000 | 4,500 | 20,000 |
| 21 | Pasteurized | 46,000 | 26,000 | 37,000 |
| 22 | Pasteurized | 31,000 | 21,000 | 35,000 |
| 23 | Pasteurized | 8,000 | 5,000 | 15,000 |
| 24 | Pasteurized | 11,000 | 1,500 | 8,500 |
| 25 | Pasteurized | 74,000 | 72,000 | 84,000 |
| 26 | Pasteurized | 31,000 | 22,000 | 62,000 |
| 27 | Pasteurized | 52,000 | 53,000 | 63,000 |

A number of pure cultures of these organisms were tested on the standard agar, and in every case it was found that visible colonies would develop when the organisms were plated from an emulsion in 1–100 sterile milk and that they would almost invariably fail to do so when plated from an emulsion in water.

When colonies did appear on the latter plates their number was always small as compared with that on the plates made from the milk emulsion. The following is the result of one of several such tests. An emulsion was made in 1–100 sterile milk and 1 cc. of this emulsion mixed with 9 cc. of sterile water and 9 cc. of sterile 1–100 milk respectively. Plates made from the two last mixtures gave:

| | |
|---|---|
| Water | (5) (3) (2) |
| 1–100 milk | (89) (69) |

The conclusions reached in this investigation are:

1. The present standard agar is not in itself a favorable medium for the growth of certain types of bacteria occurring in milk.

2. The small quantity of milk added to the medium in plating 1 cc. of a 1–100 dilution supplies the deficiency, but the amount added in plating the higher dilution does not do so.

3. More consistent results are obtained if the dilutions of the sample higher than 1–100 are made in sterile 1–100 milk instead of in sterile water.

# STERILIZING MEASURED AMOUNTS OF WATER IN THE AUTOCLAVE[1]

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It is customary for bacteriologists to measure out specific amounts of water into flasks and then to sterilize the flasks and water in the autoclave. Water is known to be lost during the process of autoclaving but the amount lost is usually considered to be so small that the errors occurring in bacterial dilutions (from this source) are small in comparison to other errors made in plating and dilution work.

To ascertain just what the loss of water in autoclaving is and just what effect this loss would have on dilutions, several tests were made. Two of these tests are reported here.

The following tables give the results of one sterilization of water in bottles in the autoclave. In putting the water into the eight ounce bottles, used in these tests, the technic was as follows: Each bottle was weighed to the nearest decigram and 99 grams or 90 grams, as desired, in excess of the weight of the bottle was placed on the opposite pan of the balance. 99 cc. or 90 cc. aliquots of distilled water were measured out by means of a 100 cc. graduated cylinder and poured into each bottle. In no case was the amount of water poured in more than 0.35 of a gram away from that desired. Water was then taken out or added so that each bottle contained the weight desired.

The bottles were sterilized for fifteen minutes under 18 pounds pressure of live steam and then the pressure was reduced at the

[1] This report is part of the work done by the author in working out and classifying errors in the plate method of enumerating bacteria. The investigations on plate methods when completed will appear as a bulletin of the Indiana Agricultural Experiment Station.

rate of one pound per minute, the door being opened thirty-five minutes after it was first closed.

*Test 1.* Two 8 ounce saltmouth bottles containing 99 grams of water and two containing 90 grams of water.

All four bottles were plugged with absorbent cotton.

The results of this test are given in table 1.

TABLE 1

*Loss of water from dilution bottles in autoclaving*

| BOTTLE NUMBER | WEIGHT OF $H_2O$ PUT IN | WEIGHT AFTER STERILIZATION | LOSS IN WEIGHT | PER CENT OF WEIGHT LOST |
|---|---|---|---|---|
| | grams | grams | grams | |
| 1 | 99 | 95.9 | 3.1 | 3.13 |
| 2 | 99 | 95.2 | 3.8 | 3.84 |
| 3 | 90 | 86.5 | 3.5 | 3.89 |
| 4 | 90 | 86.9 | 3.1 | 3.44 |
| Average | | | 3.4 | |

*Test 2.* Fourteen 8 ounce saltmouth bottles containing 90 grams of water.

Seven were plugged with absorbent cotton and seven were left unplugged. They were set in the autoclave in sets of two. The two at the rear were numbered 1 and 2, and the two nearest the door 13 and 14. Even numbers denote bottles having no plugs. Experiment conducted as in test 1. Results are given in table 2.

It is evident from these tests that the errors due to loss of water in autoclaving are considerable even with plugs in place. When a number of successive dilutions are made these errors would accumulate so that high dilutions might vary as much as 20 per cent from the dilutions desired.

On the basis of these tests it might seem advisable to ascertain by tests the amount of water that is regularly lost from bottles of water sterilized in the auto-clave under specific conditions. In some cases the loss might be constant enough so that a fixed amount of water in excess of that desired could be added to allow for losses occurring during auto-claving.

TABLE 2

*Effect of cotton plugs on loss of water in autoclaving*

| BOTTLE NUMBER | WEIGHT OF $H_2O$ PUT IN | WEIGHT OF $H_2O$ LOST | PER CENT OF $H_2O$ LOST WITH PLUG | PER CENT OF $H_2O$ LOST WITHOUT PLUG |
|---|---|---|---|---|
| | *grams* | *grams* | | |
| 1 | 90 | 2.2 | 2.44 | |
| 2 | 90 | 6.3 | | 7.00 |
| 3 | 90 | 3.7 | 4.11 | |
| 4 | 90 | 8.3 | | 9.22 |
| 5 | 90 | 3.0 | 3.33 | |
| 6 | 90 | 8.3 | | 9.22 |
| 7 | 90 | 2.9 | 3.22 | |
| 8 | 90 | 3.7 | | 4.11 |
| 9 | 90 | 3.0 | 3.33 | |
| 10 | 90 | 8.0 | | 6.69 |
| 11 | 90 | 2.4 | 2.67 | |
| 12 | 90 | 7.6 | | 8.44 |
| 13 | 90 | 2.5 | 2.78 | |
| 14 | 90 | 5.1 | | 5.67 |
| Average | | 4.8 | 3.13 | 7.51 |

Most workers place 100 cc. of water in the auto-clave expecting 1 cc. to be lost during sterilization. The results reported give an average loss of 3.2 per cent for water sterilized in bottles carrying cotton plugs. The greatest loss was 4.1 per cent and the least 2.4 per cent.

More water is evidently lost when measured amounts of water are sterilized in the auto-clave than is ordinarily allowed for.

# STUDIES IN THE CLASSIFICATION AND NOMENCLATURE OF THE BACTERIA

## X. SUBGROUPS AND GENERA OF THE MYXOBACTERIALES AND SPIROCHAETALES

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### Order V. **Myxobacteriales.** Ordo nov.

*Motile, rod-like organisms, multiplying by fission, secreting a gelatinous base, and forming a pseudoplasmodium-like aggregation before passing into a more or less highly developed cyst-producing, resting state in which the rods may become encysted in groups without modification, or may be converted into spore masses.*

There is one family only, the *Myxobacteriaceae*.

### Family I. **Myxobacteriaceae** Thaxter, 1892, p. 394

Characters those of the order.

The following generic names have been used for organisms of this group.

*Polyangium* Link, 1795
*Stigmatella* Berkeley and Curtiss, 1857, p. 70
*Chondromyces* Berkeley and Curtiss, 1857, p. 313
*Cystobacter* Schroeter, 1886, p. 170
*Myxobacter* Thaxter, 1892, p. 403
*Myxococcus* Thaxter, 1892, p. 403
*Myxobotrys* Zukal, 1896, p. 346

The genera may be differentiated by the following key:

*Key to the genera of Myxobacteriaceae*

I. Cells not transformed into coccus-like spores when encysted.
A. Rods forming free cysts in which they remain unmodified. Cysts various, sessile or borne on a more or less highly developed cystophore. Genus I. *Chondromyces*

B. Rods forming large rounded cysts, one or more, free within a gelatinous matrix raised above the substratum.....Genus II. *Polyangium*
II. Rods transformed to form definite, more or less encysted, sessile or stalked masses of coccus-like spores.....Genus III. *Myxococcus*

Genus I. **Chondromyces** Berkeley and Curtiss, 1857, p. 313

Synonyms:

*Stigmatella* Berkeley and Curtiss, 1857, p. 70
*Polycephalum?* Kalch and Cke., p. 22
*Cystobacter* Schroeter, 1886, p. 170
*Myxobotrys* Zukal, 1896, p. 346

*Rods forming free cysts in which they remain unmodified. Cysts various, sessile or borne on a more or less highly developed cystophore.*

The type species is *Chondromyces crocatus* Berkeley and Curtiss.

Genus II. **Polyangium** Link, 1795, p. 65

Synonyms:

*Myxobacter* Thaxter, 1892, p. 394

*Rods forming large rounded cysts, one or more, free within a gelatinous matrix raised above the substratum.*

The type species is *Polyangium vitellinum* Link.

Genus III. **Myxococcus** Thaxter, 1892, p. 403

*Rods slender, curved, swarming together after a vegetative period to form definite more or less encysted sessile masses of coccus-like spores.*

The type species is *Myxococcus rubescens* Thaxter

Order VI. **Spirochaetales.** Ord. nov.

Synonyms:

*Spirilloflagellata.* Krzysztalowicz and Siedlicki

*Protozoan-like in many characters. Cells usually relatively slender flexuous spirals: multiplication of cells apparently by longitudinal division in some types, by transverse division in others, or both.*

One family is recognized, *Spirochaetaceae.*

## Family I. **Spirochaetaceae** Swellengrebel 1907, p. 581

Synonyms:

*Spirochaetoidea* Dobell, 1911, p. 536
*Spironemaceae* Gross, 1912, p. 83

Characters those of the order.

The following generic names have been used for organisms of this group:

*Spirochaeta* Ehrenberg, 1833, p. 313.
*Spirochoeta* Dujardin, 1841, p. 209
*Spirochaete* Cohn, 1872, p. 180
*Treponema* Schaudinn, 1905, p. 1728
*Miscrospironema* Stiles & Pfender 1905, p. 936
*Spironema* Vuillemin, 1905, p. 1567
*Borrelia* Swellengrebel, 1907, p. 582
*Cristispira* Gross, 1910, p. 41
*Saprospira* Gross, 1911, p. 188
*Spiroschaudinnia* Sambon, 1913, p. 833.

The genera recognized may be differentiated by use of the following key:

*Key to the genera of Spirochaetaceae*

I. Usually saprophytic, free living in water.
  A. Protoplasm spirally wound around an elastic axis filament. Genus I. *Spirochaeta*
  B. Not as in (A), cross section circular..... Genus II. *Saprospira*
II. Usually parasitic.
  A. Possessing a "crest" or ridge. Parasitic in mussels. Genus III. *Cristispira*
  B. Without a crest. Parasitic in warm blooded animals. Genus IV. *Treponema*

## Genus I. **Spirochaeta** Ehrenberg, 1833, p. 313

Synonyms:

*Spirochaete* Cohn, 1872, p. 180
*Spirochoeta* Dujardin, 1841, p. 209

*Slender, spiral cells, living free, usually in water containing hydrogen sulphide, actively motile, flexuous. Flagella unknown. Anaerobic. Protoplasm is spirally wound around a flexible or elastic*

*axis filament. Volutin granules regularly present in the plasma. No differentiation of exterior. Cell circular in cross section.*

The type species is *Spirochaeta plicatilis* Ehrenberg.

Genus II. **Saprospira** Gross, 1911, p. 188

*Slender spiral cells living free in salt water, actively motile, flexuous. Cross section circular.*

The type species is *Saprospira grandis* Gross.

Genus III. **Cristispira** Gross, 1910, p. 41

*Spiral organisms known only from the crystalline style of mussels. The body of the organism is circular in cross section, more or less spirally wound and possessing a longitudinal comb or crest which does not extend quite to the tips.*

The type species is *Cristispira veneris* Gross.

Genus IV. **Treponema** Schaudinn, 1905, p. 1728

Synonyms:

*Spirochaeta* of many authors
*Spirochaete* of many authors
*Spironema* Vuillemin, 1905, p. not *Spironema* Meek, 1864
*Microspironema* Stiles & Pfender, December 2, 1905, p. 936
*Borrelia* Swellengrebel, 1907, p. 582.
*Spiroschaudinnia* Sambon, 1907, p. 833

*Cells slender, spiral, not flattened, attenuated at tips, without crest. Multiplication by longitudinal or by cross division. Parasites in warm blooded animals. Motile.*

The type species is *Treponema pallidum* Schaudinn.

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# A NOTE ON THE NATURE OF THE REACTION OF B. COLI ON ENDO MEDIUM

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There appears to exist much difference of opinion as to the factor or factors which enter into the production of typical colonies of *B. coli* on the Endo plate. Endo (1904), who developed this medium for the differentiation of *B. typhosus* from *B. coli*, states that the red colonies are due to acid produced by the *B. coli*.

Harding and Ostenberg (1912) claim that the red colonies of these organisms on the Endo plate are due to aldehyd formation, and that the gradual disappearance of the color after the first twenty-four to forty-eight hours incubation, is due to oxidation of aldehyd to acid. According to these workers, acid decolorizes Endo medium. De Bord (1917), on the other hand, claims that acid is essential for the production of red colonies; that aldehyds will not bring out the red color in the Endo medium, but acid and aldehyd will. This worker, like Levine, Weldin, and Johnson (1917) speaks of the Endo reaction as the "fuchsin-aldehyd reaction," insisting however that acid is essential for this reaction.

According to Robinson and Rettger (1916), organic acid, especially lactic acid, is the cause of the reddening of colon colonies on Endo medium, and they maintain that the later decolorization of the colonies, is due to alkali formation by the bacteria. These investigators find that a drop of lactic acid added to an Endo plate, produces a shade of red somewhat similar to that produced by *B. coli*. On the other hand, a drop of neutral

formaldehyd added to the Endo medium produces a purple-violet color.

The underlying cause of the different views expressed by these investigators, appears to be due to the variation in the strength of the reagents employed in their respective experiments. It has been observed again and again that fuchsin decolorized with sodium sulfite will behave differently toward different dilutions of the same reagent. The behavior of the fuchsin sodium sulfite solution toward strong and weak acid, or strong and weak aldehyd is such that it can not be compared. Furthermore, the quantity of sodium sulfite employed in the decolorization of the fuchsin also appears to play an important part in such experiments.

The fuchsin-sulfite combination is extremely unstable. Workers have long learned not to expect complete decolorization of the fuchsin with sodium sulfite in hot solutions, because of the dissociation under these conditions. When an Endo plate is exposed to air, the color of the medium becomes pink, probably because the sulfite in the presence of air is oxidized to sulfate, causing the fuchsin color to reappear in part. Very dilute acids also bring out the color to some extent, possibly because of the high degree of dissociation that exists in the mixture.

If inorganic acids of moderate strength be added to fuchsin decolorized with sodium sulfite, decolorization becomes even more complete. The sodium probably combines with the acid to form a salt, liberating sulfur dioxide or sulfurous acid, and causing further decolorization. Concentrated acids will reduce the color of basic fuchsin without the presence of sodium sulfite.

Organic acid added to decolorized fuchsin will cause a reappearance of the color, due possibly to a stronger affinity of basic fuchsin for the organic acid than for the inorganic sulfite, the result being the formation of acid fuchsin.

It might be said in this connection that we are dealing here with an extremely complex organic combination, and it is questionable to what extent one is permitted to draw a conclusion from a simple test tube experiment. The following few observations will be recorded with the hope that they may throw

some light on the original problem, of the cause of the red colonies on the Endo plate.

When strong aldehyd is added to decolorized fuchsin, a strong purple color is produced. Weak aldehyd produces a red color; weak aldehyd in an acid solution, a strong cherry red color. If a proper combination of aldehyd and acid be added to the decolorized fuchsin in a test tube, a metallic film will appear on the surface in a few minutes. This film, however, is not permanent, disappearing after a few hours. Acetone and alcohol in faintly acid solutions will also bring back the color to decolorized fuchsin. This, it might be added, is a matter of text-book knowledge.

It was further observed that, if glucose be substituted for lactose in Endo medium, not only are typically metallic colonies produced by the colon group, but by the typhoid-dysentery group as well. The same was also found to be true when the triatomic alcohol, glycerol, and the hexatomic alcohol, mannitol, were substituted for lactose in Endo medium.

Finally, the following experiments were performed. Two 1000 cc. Erlenmeyer flasks containing 300 cc. of 1 per cent lactose broth, and 1 per cent glycerol broth respectively, were inoculated with a fresh agar slant growth of *B. coli-communior* obtained from feces. These flasks were employed in order to permit large surface exposure. After twenty-four hours' incubation the contents of the flasks were rendered alkaline with sodium carbonate to hold back the organic acids, and distilled.

When the distillates were added to decolorized fuchsin, no change could be observed. When, however, the distillates were rendered faintly acid with acetic acid and added to decolorized fuchsin, the appearance of the color was far more marked than when the same concentration of acetic acid alone was employed. This would indicate that another substance, or possibly other substances, besides acid, are formed, when *B. coli* is grown under partial anaerobic conditions in lactose or glycerol medium, which help to bring back the color to decolorized fuchsin.

In this connection, it might be well to review briefly the chemical nature of some of the substances under discussion.

(1) The close relation which exists between alcohol, aldehyd, and acid is well illustrated by the following equations. If we take ethyl alcohol as our specific example, we have:

1. $CH_3CH_2OH + O = CH_3.CHO + H_2O$
   Ethyl alcohol — Acetaldehyd.
2. $CH_3CHO + O = CH_3.COOH$
   Acetic acid.

Thus, by oxidation, Alcohol → Aldehyd → Acid
and inversely by reduction Acid → Aldehyd → Alcohol.

It is generally believed that oxygen from the air can not effect the oxidation from alcohol to acid except through the agency of "catalyzers" or ferments.

(2) The close relation which exists between aldehydes and ketones can be seen from the following:

The oxidation of primary alcohols yields aldehydes, and the oxidation of secondary alcohols yields ketones—thus

$$CH_3.CHOH.CH_3 + O = CH_3.CO.CH_3 + H_2O$$

Secondary propyl alcohol — Dimethyl ketone or acetone.

(3) From a chemical standpoint, the carbohydrates are aldehyd or ketone derivatives of polyhydric alcohols. This is illustrated by glancing at the structural formula of a typical sugar, glucose, and the two alcohols, mannitol and glycerol.

1. Glucose = $CH_2(OH)\ CH(OH)\ CH(OH)\ CH(OH)\ CH(OH)\ CHO$
2. Mannitol = $CH_2(OH)\ CH(OH)\ CH(OH)\ CH(OH)\ CH(OH)\ CH_2OH$
3. Glycerol = $CH_2(OH)\ CH(OH)\ CH_2(OH)$

(4) The production of lactic acid from lactose by a number of organisms, including *B. coli*, has led some investigators to the opinion that the reddening of colonies produced by *B. coli* on Endo medium is due to lactic acid. It is indeed likely that the formation of lactic acid is an important intermediary step in the production of typically metallic colonies on Endo agar. However, when the alcohol, glycerol, is substituted for lactose in

Endo medium, the explanation of typical colonies produced on this medium becomes more complex. It is likely that the glycerol is first oxidized into glyceric aldehyd,

$$\underset{\text{Glycerol}}{CH_2(OH)CH(OH)CH_2(OH)} + O = \underset{\text{Glyceric aldehyd.}}{CH_2(OH)CH(OH)CHO} + H_2O$$

and this in turn is converted into lactic acid. It will be recalled that Embden and his co-workers (Hammarsten, 1914) have shown that in the formation of lactic acid from glucose by enzymes, glyceric aldehyd is one of the intermediary products.

Grey (1913), who has investigated the products of anaerobic glucose decomposition by *B. coli-communis*, finds that this organism produces lactic, acetic, formic, and succinic acids, alcohol, and acetaldehyd from this carbohydrate. Mendel (1911) finds also the presence of acetone among glucose decomposition products of *B. coli*.

To recapitulate the observations discussed above:

1. Acid, aldehyd, acetone and alcohol in proper dilution and combination cause a reappearance of the fuchsin color of a decolorized fuchsin-sulfite solution.

2. When glucose, mannitol and glycerol are substituted for lactose in the Endo medium, typically metallic colonies are produced by the entire typhoid-colon group.

3. *B. coli-communior*, when grown in lactose and glycerol broth under partially anaerobic conditions, produces another substance—or possibly substances—besides acid, which bring back the color to decolorized fuchsin, when faintly acidified.

In view of these observations and those of the workers mentioned above, it would appear that the red colonies which *B. coli* produces on the Endo plate are due to a number of substances—including lactic and other organic acids, traces of aldehyd and possibly also acetone and alcohol. The latter of these substances are volatile and therefore evaporate on exposure, the result being that the non-volatile fuchsin remains behind and we thus have the metallic film.

The possible criticism that *B. coli* breaks down glucose into the various substances named above, only when grown anaerobi-

cally, but not during the aerobic growth on Endo agar, does not appear to hold. Organisms growing on the surface of agar must indeed have the ability to grow in the presence of air in order to get a start, so to speak. But once the start is gained the surface organisms alone grow aerobically, while those organisms below the surface of the colony are probably growing in no less an anaerobic environment than those growing below the surface in carbohydrate broth.

Observations of the gradual disappearance of the metallic film of colon colonies on Endo agar corroborates this view. It is reasonable to assume that the organisms below the metallic film of a colon colony, are growing in an anaerobic environment. These organisms, after twenty-four to forty-eight hours incubation, proceed to break down the nitrogenous bodies of the nutrient media into ammonia and amines (Robinson and Rettger), creating a condition favorable for the re-solution of the metallic fuchsin and final decolorization. In other words, we have here a chemical change produced by *B. coli* growing *anaerobically* in apparently aerobic colonies.

As further evidence that the metallic sheen of colon colonies on Endo medium results from the evaporation of volatile substances produced during the metabolism of these organisms, the following two observations are cited.

1. If *colon bacilli* are grown anaerobically in Endo agar, the colonies are red but not metallic. Under anaerobic conditions, *B. coli* breaks down carbohydrate into various acids, aldehyd, and possibly acetone and alcohol (Grey and Mendel). These substances bring out the color of decolorized fuchsin and the colonies become red. The fuchsin, however, remains in solution, because the volatile substances can not evaporate. The same colonies exposed to air become metallic, because surface evaporation permits the disappearance of the substances which hold the fuchsin in solution.

2. The metallic sheen of a given colon colony is markedly pronounced when grown in such a manner that evaporation can take place on a large scale. Thus, if *B. coli* be inoculated on Endo plates and on Endo agar slants, the metallic film, after a

given period of incubation, will be more pronounced on the Endo agar slant than on the Endo plate. This is explained as due to the greater opportunity for evaporation from the slant han from the inverted plate.

There are a large number of red colonies on Endo medium which workers recognize to be of non-colon types, These colonies, as a rule, possess a red shade that is somewhat different from that of *B. coli* and they never possess a metallic film. It is possible that some organisms cause the production of red colonies by reducing sodium sulfite to hydrogen sulfide, thus liberating the fuchsin color. It is more likely, however, in view of the ease with which bacteria attack carbohydrates, that these red colonies are produced by the conversion of lactose to some non-volatile acid, most probably lactic acid. This acid has a tendency to absorb moisture from the air, and such moisture on the surface of the colony would tend to keep the fuchsin in solution, rendering the colony red but not metallic.

## CONCLUSIONS

1. Chemical and physical factors enter into the formation of typically metallic colonies of *B. coli* on Endo agar.

2. During the growth of these organisms on Endo medium, they adsorb the various soluble substances contained in the medium and proceed to break down the lactose first (Kendall).

3. After ten to fifteen hours incubation, the trace of lactose adsorbed, is probably transformed into lactic and other organic acids, and the colony is colored red.

4. On further incubation, the organic acids are probably reduced by the bacteria to aldehyd and alcohol, which volatilize from the surface, leaving the non-volatile fuchsin behind, thus producing a metallic sheen.

5. The carbohydrate being disposed of, the organisms proceed to attack the nitrogenous materials. Ammonia and other substances are produced in which the fuchsin goes back into solution, and ultimate decolorization takes place.

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# A SIMPLIFIED CONFIRMATORY TEST FOR B. COLI

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When the presumptive test for *B. coli* has been established by gas production in lactose broth, we have, according to the committee on Standard Methods of Water Analysis (1917), two more steps to carry out before the presence of *B. coli* is completely confirmed. Transplantations are made from the lactose fermentation tubes showing gas, to Endo plates and typical colonies sought for after twenty-four hours incubation. If such colonies be found, the test for *B. coli* is "partially confirmed." One or more of these colonies are again inoculated in lactose fermentation tubes and examined for gas production after incubation. The presence of gas in these fermentation tubes renders the presence of *B. coli* completely established.

During our investigation of the nature of the reaction of *B. coli* on Endo medium (1918), it was observed that colonies of *B. coli* are more typically metallic when grown on Endo agar slants than on inverted Endo plates. This finding has led to the view that possibly test tube slants containing Endo medium, might advantageously be substituted for Endo plates in the confirmatory test for *B. coli* in water.

It appeared also that if the inoculation of these organisms in an Endo tube be made both in the butt and on the slant, we would have, after proper incubation, typically metallic colonies on the slant and a practically anaerobic condition in the butt with gas production and reddening of the medium. We should thus have the "partially confirmed" and "completed" tests for *B. coli* in one procedure.

When testing out the growth of a number of strains of *B. coli* under these conditions, it was observed that while the colonies on the surface of the slant were typically metallic, the organisms growing in the butt, although showing gas production, did not always redden the medium during the first twenty-four hours of incubation. This fact, however, was not found to interfere with our intention of utilizing the Endo tube rather than the plate for the confirmatory test for *B. coli* in water. When waters sent to this laboratory for examination showed gas formation in lactose broth fermentation tubes, straight wire inoculations were made from these tubes, to Endo tubes, extending the wire first into the butt, then spreading the tip of the wire over the slant. Endo plates were also inoculated as a check. The results were always constant. When colonies of *B. coli* were present on the Endo plates, typical colonies were present also on the slant, with gas production in the butt. Occasionally, after a given period in the incubator, the colonies on the plate would require further incubation, while those on the slant would be sufficiently metallic to render the test complete.

This constancy of results, combined with the saving of time and material by this new procedure, led to the complete substitution of Endo agar slants for Endo plates in the confirmatory test for *B. coli*.

The following procedure also, has been employed as a confirmatory test for *B. coli:* A loop from the lactose fermentation tube showing gas, is spread over the surface of an empty Petri dish. A tube of Endo agar is now melted in a water bath, cooled to 40°C. and poured over the surface of the dish. When the Endo agar is congealed, a loop from the same fermentation tube is smeared over the surface of the agar. After incubation, if *B. coli* be present, typical colonies will appear on the surface and red colonies with gas bubbles, below the surface of the agar. Thus an aerobic growth and a practically anaerobic growth is obtained in one test.

However, no marked advantage is gained when employing this procedure over the simple Endo tube method described above.

Occasionally *B. coli* are found in water which form non-typical colonies on Endo medium, i.e., colonies without a metallic sheen. Under such conditions, at least two colonies considered to be most likely *B. coli*, are transferred into lactose broth fermentation tubes (Standard Method) and gas formation observed after twenty-four and forty-eight hours. The presence of gas in these tubes constitutes a positive test, while the absence of gas at the end of forty-eight hours constitutes a negative test for *B. coli*. In view of the difficulty in differentiating non-typical colonies of *B. coli* from non-colon colonies on the Endo plate, it is thus possible, by inoculating non-colon colonies into fermentation tubes, to miss the detection of *B. coli*. On the other hand, when such colonies are grown in Endo tubes, we have, aside from their growth on the slant, also gas formation in the butt. We thus have two factors which help to establish the presence of the organisms.

There is, of course, a remote possibility that we might have colon-like colonies on an Endo agar slant and gas formation in the butt, due to an anaerobe instead of *B. coli*. When such a condition is suspected, inoculations of several colon-like colonies in lactose broth fermentation tubes, should be resorted to. It might be mentioned however, that in our work with anaerobic lactose fermenting organisms, obtained from waters of the southeastern area of the United States, in not a single instance did such organisms produce gas in the butt of Endo tubes.

Another important advantage of the substitution of the Endo tube for the Endo plate in confirmatory tests for *B. coli*, lies in the fact that tubes can be kept in the ice-box from three to four weeks without deterioration. Russell (1912) and Robinson and Rettger (1916) have suggested that Endo medium for typhoid work be first stored in tubes, and plates poured from these when ready for use. These investigators found that this medium keeps well in tubes from two to three weeks in the ice-box.

It is well known that Endo plates, even if kept in the dark, will begin to redden after forty-eight hours. This appears to be due to the large surface exposed to air, which brings about the oxidation of the unstable sodium sulfite to sulfate. Tubes con-

taining Endo medium were kept on the working table exposed to direct light for close to two weeks without any appreciable change in the butt; the slant, however, became red after twenty-four hours. This is explained by the fact that air can not come in direct contact with the medium in the butt; no oxidation of the sulfite takes place, and the medium remains unchanged. Endo tubes have been kept in this laboratory for four weeks in the ice-box without any apparent deterioration.

It might also be added that unless a sufficient number of previous examinations of a given water have established the absence of *B. coli* in it, we do not wait for gas production in lactose broth before inoculating in Endo tubes. It was observed again and again that if waters showing a high bacterial count be inoculated in lactose broth fermentation tubes and after about fifteen hours incubation—when there is no sign of gas production—inoculations made in Endo tubes, typically metallic colonies on the Endo slant with gas production in the butt often develop simultaneously with gas in the original lactose fermentation tubes. Thus, at the appearance of the "presumptive test" we would have the "completed test" also. These findings have led to the application of this procedure to all waters coming into this laboratory in which the absence of *B. coli* has not been established by previous tests.

The question of the strength of agar to be employed in the Endo tube media is of some importance. In a comparative study of Endo media containing 1.5 per cent and 3 per cent agar, no marked variations were observed in the amount of gas produced in the butt. A strong gas producing organism would usually produce much gas, while a weak one, would produce only a few bubbles, in both cases.

It was observed also that colonies of *B. coli* have, as a rule, a stronger metallic film, when grown on Endo medium containing 1.5 per cent agar than 3 per cent agar. The difficulty with the former, however, is the fact that the surface of the slant is apt to be moist. This moisture may prevent the formation of isolated colonies of *B. coli*—and such colonies are desirable for the final isolation of these organisms.

Of late we have been employing Endo medium of 2 per cent agar, with good results. The surface of the slant is, as a rule, dry and there is no difficulty in obtaining isolated colonies.

There also exists some difference of opinion as to the amount of fuchsin to be employed in Endo medium for colon work. In this laboratory, 0.2 per cent of a saturated alcoholic solution of basic fuchsin in agar is employed for the isolation of *B. typhosus* from feces. This amount of fuchsin approaches that suggested in the "Standard Methods," and although sufficient for typhoid work, is too weak for the detection of *B. coli* in water. Hasseltine (1917) comes to the same conclusion. 0.5 cc. and 0.4 cc. of a saturated alcoholic solution of basic fuchsin per 100 cc. of agar have been employed with good results. Our findings would indicate that 0.4 per cent of fuchsin is sufficient for colon work.

The method of sterilization suggested by Robinson and Rettger (1916) has been employed for this medium. Stock quantities of 2 per cent agar, titrated to 0.2 to phenolphthalein are kept on hand. Just before tubing, proper quantities of sterile lactose, fuchsin, and fresh sodium sulfite are added to the agar previously melted in the Arnold. The tubing is carried out with care to prevent undue contamination. The tubes are then placed in the hot autoclave and sterilized from five to seven minutes at 10 pounds pressure. After sterilization, the tubes are slanted and covered with a towel to keep out the light, after which they are placed in the ice-box and are ready for use.

Whether or not typically metallic colonies are produced on Endo medium by organisms not related to the colon group has been under investigation for some time. When glycerol or glucose is substituted for lactose in Endo medium, other organisms beside *B. coli*, produce red colonies with a metallic film. When, however, regular Endo medium is employed with lactose as the carbohydrate, no metallic colonies were observed except by members of the colon group.

Fifty Endo plates were exposed to air and were then incubated; twenty-five plates at 37°C., and twenty-five plates at room temperature. Observations were made every day for two

weeks. A large number of red colonies were observed, but none with a metallic sheen. One can not, however, draw any definite conclusions from this small number.

## RÉSUMÉ

It is suggested that:

1. In the confirmatory test for *B. coli*, Endo slants be employed instead of Endo plates.

2. The inoculations from the lactose fermentation tubes be made in the butt as well as on the slant of the Endo tubes, to permit aerobic and anaerobic growth.

3. In the case of unknown waters, inoculations be made from the fermentation tubes into Endo tubes after about fifteen hours incubation, even if there be no gas present in the fermentation tubes at that time.

The advantages of the proposed modifications are:

1. The production of more typical colonies of *B. coli* on the Endo slant than on the Endo plate.

2. The detection of aerobic and anaerobic growth in the same test.

3. Endo agar tubes can be kept for three to four weeks without deterioration.

4. The inoculation from the presumptive fermentation tubes to Endo agar tubes before the production of gas in the former will often save several days time in establishing the presence of *B. coli* in an unknown water.

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# THE DIFFERENTIATION OF LACTOSE FERMENTING ANAEROBES FROM B. COLI

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The procedure commonly employed for the differentiation of anaerobes from *B. coli* consists largely of a repetition of the presumptive and confirmatory tests of the committee on Standard Methods, (1917). Thus, according to Frost, (1916).

If no typical colonies develop within twenty-four hours on Endo plates made from fermentation tubes showing gas, further effort is made to recover *B. coli* as follows: (1) One or more colonies are transferred to lactose broth fermentation tubes. The formation of gas demonstrates the presence of *B. coli*; (2) Plates are again made from the original fermentation tube; (3) A transplant is made from the original fermentation directly to another lactose broth tube, if steps (1) and (2) have both failed to recover *B. coli*, plates are now made from this transplanted culture; (4) At the same time a transfer is made from this tube directly to a third fermentation tube.

If all the above procedures fail to recover *B. coli* and gas is still formed in this fermentation tube, the inference is that gas in the preliminary test was due to an anaerobe.

If an anaerobic lactose fermenting organism be inoculated in the butt and on the slant of an Endo tube, we should theoretically have reddening and gas production in the butt and no growth on the slant. It was observed, however, that when gas formation in lactose fermentation tubes was due to anaerobes, transplantations from these tubes to Endo tubes showed no growth at all—either on the slant or in the butt of these tubes. It seems that aside from the inability of anaerobic organisms to grow aerobically on the slant, the fuchsin-sulfite

combination is sufficiently toxic to prevent their growth even in the butt.

This lack of growth in Endo tubes after inoculations from lactose broth fermentation tubes showing gas, has helped us to differentiate anaerobic lactose fermenting organisms from *B. coli*, in a large number of cases.

Frequently, however, when gas production in lactose broth is due to an anaerobe, other organisms capable of aerobic growth are found in the medium. Under these conditions, after transplantations from fermentation tubes to Endo tubes, a growth would occasionally appear on the slant, but as a rule, would be recognized without difficulty to be of non-colon type.

As a further means for differentiating lactose fermenting anaerobes from *B. coli* the following procedure may be employed:

A loop from the lactose fermentation tube showing gas, is smeared on the bottom of a Petri dish. A tube of Endo agar is melted in a water bath, cooled to about 40°C. and poured over the surface of the plate. After the Endo agar is congealed, another loop from the same fermentation tube is now spread over the surface of the agar. We thus have, after incubation, an aerobic growth on the surface and a practically anaerobic growth below the surface of the Endo agar.

If *B. coli* be present, typical or semi-typical colonies will appear on the surface; also red colonies, and as a rule, gas bubbles will appear below the surface of the medium. If on the other hand, the gas produced in lactose broth is due to an anaerobe, typical colon colonies are of course, never present; the colonies below the surface of the agar are, as a rule, uncolored; neither are gas bubbles observed below the surface of the medium.

It is well to keep in mind that while *B. coli* represent a definite group of organisms with well defined characteristics, the anaerobes found in water vary widely both in traits and morphology. It is likely that geographic and climatic conditions will favor the growth of certain types of anaerobes in one area, and of different types of anaerobes in another area of the country. A procedure, therefore, which would help differentiate anaerobes from *B.*

*coli*, applied for waters of the southeastern area of the United States, may not be applicable to waters of the northern and western areas of the country. Thus the anaerobes which we have been able to observe, have not in a single instance produced gas in the butt of Endo tubes. On the other hand, it is likely that anaerobes found in waters in other parts of the country might possess this ability.

The ideal procedure for the differentiation of anaerobic lactose fermenting organisms from *B. coli* should not only establish the absence of *B. coli*, but permit the isolation of the anaerobe as well. Simonds and Kendall (1912) and Jones (1916) have described good procedures for the isolation of anaerobes in pure culture. The application of these methods to the isolation of anaerobes found in water, would provide valuable scientific data. The practical worker, however, would probably find the isolation of anaerobes by either of these methods not commensurable with the work involved, since the mere isolation of a lactose fermenting anaerobe would not rule out the possible presence of *B. coli*, and it is this fact that the worker is primarily interested in.

It might be added that it is questionable whether "anaerobes" is a proper term to apply to non-colon lactose fermenting organisms found in water. We have twice transplanted spreading growths from Endo plates into lactose fermentation tubes which resulted in gas formation in these tubes after incubation. These growths on the Endo plates could readily be distinguished as non-colon types. It is evident, however, that not all non-colon lactose fermenters, are anaerobes. Meyer (1918) has recently reported the finding of a lactose fermenting organism in water, capable of growing aerobically.

## RESUMÉ

1. Anaerobic lactose fermenting organisms found in waters of the Southeastern area of the United States, appear to be unable to produce gas when grown anaerobically in Endo medium. In accordance with this finding, the differentiation of anaerobes from *B. coli*, is accomplished as follows:

2. Straight wire inoculations are made from the lactose fermentation tube showing gas, into the butt and on the slant of Endo tubes. After incubation (1) *B. coli* produce typical or semi-typical colonies on the slant and gas in the butt. (2) Anaerobes neither grow on the slant nor produce gas in the butt of these tubes.

3. Employing the same medium, another procedure for the differentiation of anaerobes is described in the text, wherein plates are employed instead of tubes.

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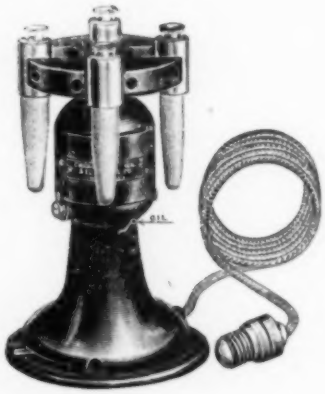

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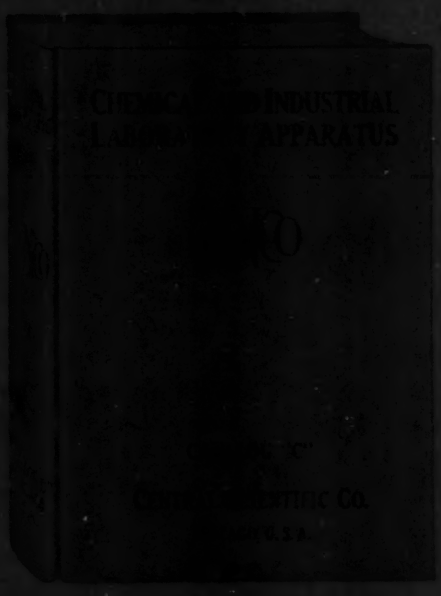


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